

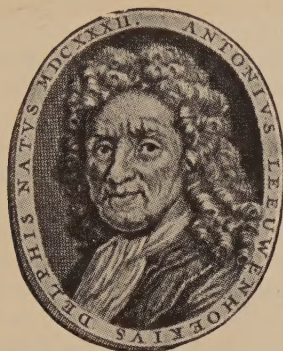
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ANTONIE VAN LEEUWENHOEK

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THE DISTRIBUTION OF CHROMATIN IN BUDDING YEAST CELLS ¹⁾

by

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DAN O. McCLARY**

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Recent advances in the cytology of microorganisms have depended largely on procedures which remove ribosenucleic acid from the cell without removing desoxyribosenucleic acid. Hydrolysis with hydrochloric acid has been exploited for this purpose with considerable success by ROBINOW (1945), BISSETT (1950), DELAMATER (1950) and many others and has also served as a means of preparing the cells for the Feulgen reaction. The difficulty in using acid hydrolysis on yeast cells is that this procedure usually destroys the vacuole. The extraction of RNA from the cells with 10 per cent perchloric acid preserves the vacuole and has been used for the procedures reported in this paper.

OGUR, MINCKLER, LINDEGREN and LINDEGREN (1952) have shown that perchloric acid removes practically all the RNA but only an insignificant amount of the DNA from the yeast cell. An important aspect of the treatment of the cells with perchloric acid is that it removes all the metaphosphate and thus eliminates the possibility of confusing this basophilic material with chromatin, a difficulty which might assume serious proportions, especially with yeasts. Digestion with perchloric acid is superior, therefore, to digestion with ribonuclease since RNAase would not remove metaphosphate. The Giemsa stain was chosen because of its high staining intensity; yeast cells subjected to the Feulgen procedure stain very lightly.

¹⁾ This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service C-2140 and from the Illinois Division of the American Cancer Society.

MATERIALS AND METHODS.

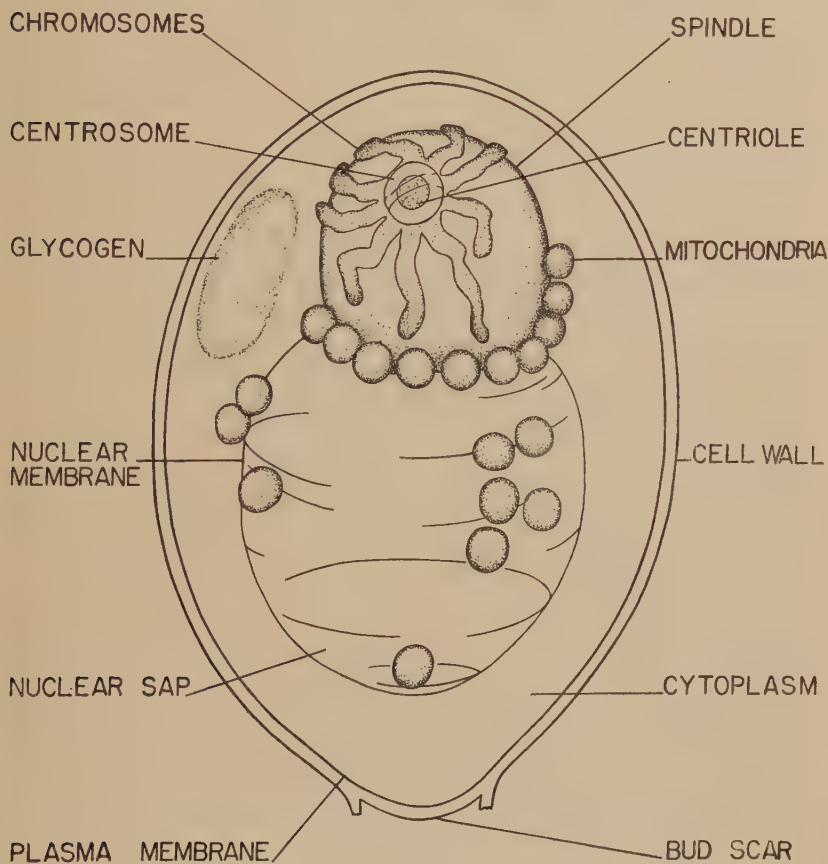
The culture used in these experiments is a tetraploid strain of *Saccharomyces* obtained through the mating of the Carbondale cultures 11294 and 11296 (LINDEGREN and LINDEGREN, 1951).

The entire cell content of fresh slants (not over 7 days old) was used to inoculate flasks containing 50 ml of the following medium: glucose, 20.0 g; Basamin (Anheuser-Busch), 5.0 g; MgSO_4 , 0.5 g; KH_2PO_4 , 2.0 g; $(\text{NH}_4)_2\text{SO}_4$, 2.0 g; distilled water, 1000 ml. The culture was grown for 24 hours. The sugar in the medium is consumed early and the cells cease to bud and settle into a nondividing (dormant) stage during which a low state of metabolism is maintained. The dormant cells were centrifuged and inoculated into a medium of the following composition: glucose, 8.0 g; KH_2PO_4 , 0.8 g; $(\text{NH}_4)_2\text{SO}_4$, 3.0 g; CaCl_2 , 0.2 g; MgSO_4 , 0.17 g; Basamin, 4.0 g; and distilled water, 400 ml. The centrifuged cells were placed in a cylinder of the above medium and aerated by a sparger. Samples were taken at zero time and every 15 minutes for a period of 3 to 4 hours, or through two complete budding cycles. The volume of the medium in the cylinder was maintained by the addition of fresh medium as required. Samples were taken in small tubes and all procedures carried out in tubes to avoid drying the cells.

After the removal of the samples they were centrifuged and washed once with distilled water. The cells were then fixed for two hours in a modified Carnoy solution of the following composition: 70 per cent ethyl alcohol, 90 parts; formalin, 5 parts; glacial acetic acid, 3 parts. After fixation the cells were centrifuged, washed once in distilled water and placed in 10 per cent perchloric acid overnight at 4°C. After the extraction with perchloric acid, the cells were centrifuged, washed twice in distilled water, and stained in Giemsa solution in a phosphate buffer at pH 6.8 for 30 minutes. The cells were then centrifuged, washed once in the buffer solution which was decanted. The small amount of buffer solution remaining in the tubes permitted the examination of the cells in the buffer solution and avoided the shrinkage inevitably produced by drying.

The Carnoy-perchloric-Giemsa procedure reveals considerably more chromatin than the Feulgen procedure because Giemsa stains both the DNA and its associated protein (JACOBSON and WEBB, 1952); in Giemsa preparations a larger amount of the chromosomal material is stained, therefore, than by the Feulgen method. It is

especially important to note that Carnoy-perchloric-Giemsa preparations are free of metaphosphate, which occurs on the chromosomes and is stained by Giemsa. The photographs were made on Panatomic X film, photographed through a 560 mu interference filter which produces nearly monochromatic illumination.



Text-Figure 1.

A diagrammatic representative of the dormant vegetative yeast cell.

The dormant yeast cell. In the dormant yeast cell (Text-figure 1) the cell wall surrounds the protoplast, which is enclosed in the plasma membrane. The latter is occasionally revealed in fixed preparations. The nuclear apparatus comprises the hyaloplasm and the spindle enclosed in the nuclear membrane. The chromosomes oriented to the centriole form a filamentous network

over the surface of the spindle. The stable position of the nucleus in the cytoplasm and the relative immobility of the mitochondria indicate that the cytoplasm is quite viscous. The mitochondria have often been considered sites of synthetic activity but it seems equally, if not more probable, that they are storage organelles. In yeast the mitochondria have a ribose nucleoprotein center (CASPERSSON and BRANDT, 1941) enclosed in a lipoidal envelope (LINDEGREN, 1949). In dormant cells the lipoidal envelope is conspicuous but breaks down as metabolic activity develops, suggesting that the lipid acts as a hydrophobic barrier separating the enzymes stored in the mitochondria of dormant cells from contact with the hydrophilic cytoplasm. Counts of mitochondria in *Saccharomyces* by MUNDKUR (1953b) revealed an exceptionally high frequency of even-numbered classes and a deficiency of odd-numbered classes. It may be inferred that mitochondria multiply by binary fission; this view is not in contradiction to the view that they may be storage organelles; as soon as one reaches a maximal size, it divides to form two. Only one or two are needed in a bud to insure the daughter cell's eventual content of the normal number.

In the growing cell the chromosomes extend into the nuclear vacuole. The major fraction of the cell's metabolic activity probably occurs in the cytoplasm where protein is most concentrated, with only a minor fraction occurring in the nuclear vacuole. The occurrence of metaphosphate in the nuclear vacuole would probably precipitate protein and usually restrict enzymatic activity either to the body of the chromosome or the nucleolus. The direct control of cytoplasmic metabolism exercised by the nucleus may result from the synthesis of small molecules within the nuclear vacuole (presumably at the gene-loci) and their accumulation in the nucleolus (SCHULTZ, CASPERSSON and AQUILONIUS, 1940) with subsequent passage into the cytoplasm to effect either the activation or synthesis of enzymes.

The chromatin in the resting cell. In dormant cells (collected at zero-time) the chromatin is normally on the spindle (Text-figure 1; LINDEGREN and TOWNSEND, 1954) and the nucleoplasm is clear (Fig. 1). The chromatin of the dormant cell forms a network of small fibers (Fig. 2) or condensed rods (Fig. 3) distributed over the outer surface of the spindle. Occasional zero-hour cells are found in which growth has been arrested when the chromosomes are extended into the vacuole, but these are exceptional

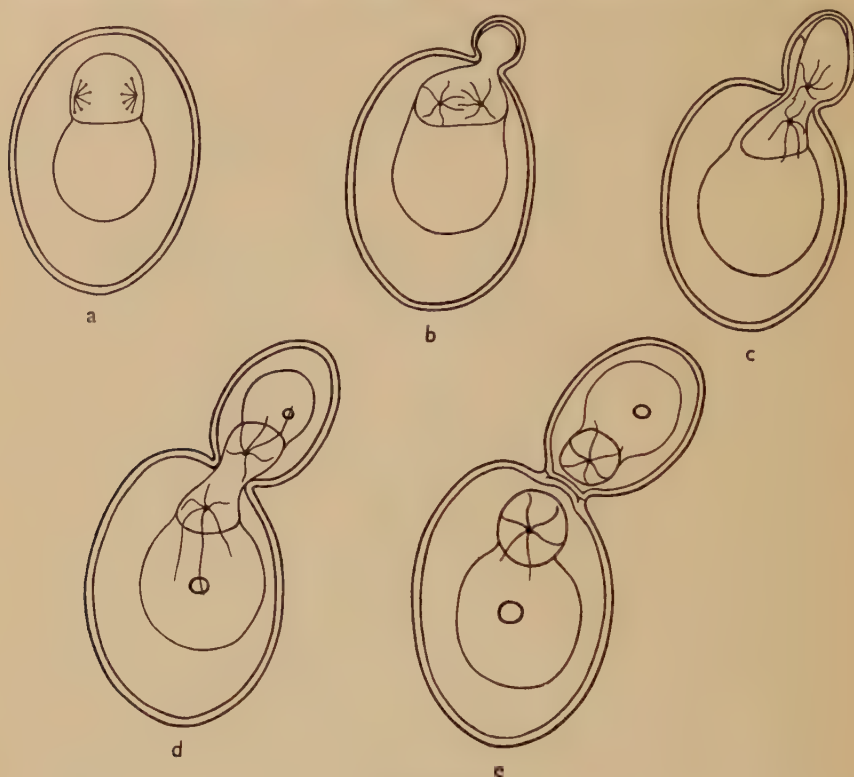
(Fig. 4). The large cells of the tetraploid and the improved fixation has led to the following interpretation: chromosomes in dormant cells lie on the surface of the spindle either in the form of a network of long, slender filaments, or more condensed rods, which often appears to radiate from a central point (Fig. 2). The designation "centrochromatin" (LINDEGREN, 1952; TOWNSEND and LINDEGREN, 1953) is superfluous, since it was intended to distinguish centrochromatin (as heterochromatin) from chromosomal chromatin (vacuolar euchromatin).

In most preparations originating by other procedures, the chromatin is aggregated into a condensed layer (or cap) closely applied to a small portion of the external surface of the spindle and giving a typical "half-moon" appearance when viewed from one side. It is inferred that the condensation of the chromatin into a single mass is the result of aggregation of the chromatin network. HENNEBERG (1926) showed that the "crescent" appears without staining in cells mounted in dilute acetic acid, and TOWNSEND and LINDEGREN (1953) found that such cells are viable. The coagulation of the chromatin to form the crescent may occur, therefore, in living cells without impairing their viability. Since the crescent always lies in contact with the nuclear vacuole, we have inferred that the chromosomes (attached to the spindle, and extending into the vacuole) shrink back onto the spindle when they are coagulated. The chromosomes in dormant cells normally appear to be condensed on the spindle in an uncoagulated state.

The centriole. A single, dense, spherical body which stains deeply with Giemsa is seen in some preparations associated with the chromosomes on the surface of the spindle (Fig. 5) surrounded by a clear area. The stained granule has been provisionally identified as the centriole in agreement with RENAUD (1938). The clear area surrounding the granule is designated the centrosome, an appellation previously applied by LINDEGREN (1952) to what is now called the spindle.

Mitosis. The time of mitosis appears to depend both on the physiological condition of the dormant cell and the stage of the growth cycle. Under the conditions which have been set up in these experiments, the first mitosis after cells break dormancy usually occurs from 30 to 45 minutes after zero-time, before the first bud appears. As cells progress into the second or third division of the growth cycle, mitosis may occur later with regard to budding. Cells

dissimilated more than twenty-four hours may undergo a longer lag before the first division of chromatin occurs. Figures 6 and 7 show the division of the chromatin into two separate masses on the



Text-Figure 2.

A diagram interpreting current data concerning the different steps involved (1) in mitosis and (2) in the partitioning of the products of mitosis between the mother and the bud. (a) Anaphase of mitosis occurs on the spindle before the formation of the first bud (Figs. 6 and 7). (b) The formation of the bud by the extrusion of the spindle occurs without the transfer of chromatin into the bud (Figs. 8 and 9). (c) As the bud enlarges the two genomes, each presumably held together by its respective cell-center, form a confluent mass over the surface of the spindle (Fig. 10) with the chromosomes of the genome of the mother cell spinning out into the vacuole of the mother cell (Fig. 11). The process directly from the bud vacuole into the bud initiates the new bud vacuole (LINDEGREN, 1951). (d) The bud enlarges and elongation of the spindle separates the two genomes (Figs. 22 and 23). A nucleolus appears in the vacuole. (e) As the bud approaches maturity and the time for the next mitosis approaches the chromosomes are retracted from the vacuole to the surface of the spindle (Figs. 23 and 24).

spindle. Although chromosome counts could not be attempted, LEVAN (1947), KATER (1927), LIETZ (1951), SINOTO and YUASA (1941), and others have described discrete chromosomes in anaphase. According to LEVAN, at metaphase, "The chromosomes form a ring on a hollow spindle." We have inferred that the mitosis which produces two complete chromosome sets occurs on the surface of the solid spindle and is then followed by partitioning of the two genomes between mother and bud according to the diagram shown in Text-figure 2.

It was shown by LINDEGREN (1951) that the bud is formed by an extrusion of the spindle into the cell wall (Figs. 8 and 9). This corrected an earlier view (LINDEGREN, 1945) that the bud was initiated by the process from the mother vacuole which initiates the vacuole in the bud. The present view is represented in Text-figure 2. The tube passing from the vacuole of the mother cell to originate the vacuole in the bud (JANSENS and LEBLANC, 1898; LINDEGREN, 1945) does not provide a channel for the passage of the chromosomes into the bud as LINDEGREN and LINDEGREN (1946) proposed but serves the function of establishing the vacuole in the bud. The bud appears after the division of the chromatin before chromatin passes into the bud. Shortly thereafter, a hollow cone of chromatin covers the spindle (Fig. 10). When the bud is well-developed (60 to 120 minutes from zero-time) the chromatin extends into the vacuole of the mother cell (Figs. 11—19). The chromatin in the vacuole remains polarized to the spindle in the condition characteristic of the fungal nucleus. Separation of the chromatin into two masses — one in the vacuole and the other on the spindle is seen frequently after acid hydrolysis (Fig. 14) but less frequently when Carnoy fixation is followed by the volutin stain. We have inferred that the separation is due to the disintegration of the chromosomal threads and coagulation of the chromosomes in the vacuole. As the chromatin progresses into the bud, it appears in the form of two hollow cones connected by their apices conforming to the external shape of the hour-glass form of the spindle (Figs. 17, 18, 20 and 21). The view that the chromatin comprises two diverging chromatinic rods stretching from the mother into the daughter cell which divide transversely after the bud is formed (BADIAN, 1937; LINDEGREN, 1949, p. 6–7) arose from the fact that a central optical section of the hour-glass shaped spindle covered with chromatin revealed an unstained clear center with two streaks of chromatin defining the

outer edges of the hollow cones of chromatin (LINDEGREN and RAFALKO, 1950, Figs. 1-5). The chromatin passing into the bud is separated from the chromatin of the mother cell in Figure 23, although the bud has not yet been cut off. After the cells are separated, each contains its full complement of chromatin (Fig. 24), and the nucleus (vacuole and spindle) rotates (LINDEGREN, 1945) to orient the spindle end of the nucleus distal to the point of union of mother and bud.

The nucleolus. During the period of rapid division the nucleolus appears in the vacuole; it is this organelle which definitively establishes the vacuole as a nuclear structure (Figs. 25 and 26). The nucleolus does not stain with Giemsa after the RNA has been removed from the cell but is visible as a perfect sphere in the vacuole. The nucleolus is often coated with metaphosphate in cells growing in a medium rich in phosphate. When cells are growing rapidly in the second or third budding cycle the nucleolus often divides at the same time as the chromosomes, and one nucleolus appears in the mother and another in the bud vacuole. The nucleoli are seen clearly in cells fixed in Carnoy and stained with the volutin stain (Figs. 26 and 27).

Correlation of metaphosphate and chromatin. The metaphosphate stain following Carnoy fixation, but without perchloric extraction, reveals essentially the same forms as the Giemsa stain, and, in addition, often shows a well-stained nucleolus, suggesting that the Giemsa-stained chromatin and the nucleolus may be covered with metaphosphate when an excess of phosphate is available. The chromatin together with its metaphosphate covering is transported into the bud vacuole along the bridge supplied by the spindle (Fig. 23) rather than through the tube which passes into the bud to initiate the bud vacuole.

DISCUSSION.

The Carnoy-perchloric Giemsa method. The Carnoy-perchloric-Giemsa procedure has three advantages over most others:

- (1) The results are duplicable and all the cells in a preparation at a given stage of growth show similar structures. This has not been our experience with other procedures. Only a few cytologists who have established reputations with conventional materials have

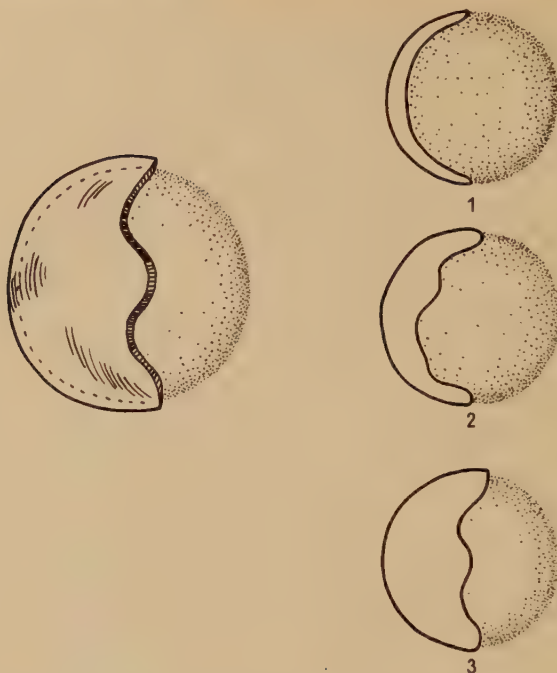
worked with yeasts, and the comment of a man of LEVAN's standing is especially significant. He states "The fixation and staining both of untreated yeast and cf-cells are, however, always whimsical. The casual state of the culture and other factors out of control seem to play an important role There is a strong tendency of the chromosomes at all stages to stick together into one or two bodies. The low chromosomes number earlier published for yeast (e.g., BADIAN, 1937; SINOTO and YUASA, 1941) may have been influenced by such fusions."

(2) The Carnoy-perchloric-Giemsa procedure preserves the vacuole.

(3) It does not cause the chromatin to clump into dense masses or thin ribbons as often occurs with other fixatives. Schaudinn-hemotoxylin preserves the vacuole but (LINDEGREN, 1951) coagulates the chromatin to a thin layer on the spindle.

The relation of the chromatin to the spindle. MUNDKUR's (1953a) view that the chromatin is homogeneously dispersed in a vesicle (other than the vacuole) is contradicted by our photographs. Many workers have assumed that the "half-moon" of condensed chromatin associated with the spindle is the "normal" distribution of chromatin, but LINDEGREN and RAFALCO (1950) showed that the chromatin is outside the spindle in the form of numerous tiny granules and that the spindle itself is solid and does not contain chromatin. This view was corroborated by LINDEGREN (1951), TOWNSEND and LINDEGREN (1953) and LINDEGREN and TOWNSEND (1954). The aggregation of chromatin on the surface of the spindle is seen in HENNEBERG's (1915) early drawings in which he designates the chromatin associated with the spindle as the "denser" "Kernkopf" and the spindle as the "less dense" "Kernleib"; from his drawings it seems clear that the Kernkopf is external to the Kernleib. LIETZ (1951) in one of the more recent publications on this subject has concluded that the spindle (HENNEBERG's Kernleib) is a vesicle and that the associated chromatin (HENNEBERG's Kernkopf) is contained within the vesicle but his figures (Abb. 1, c) are more simply interpreted as a solid spindle coated with a cap of dense chromatin as indicated in Text-figure 3. LEVAN (1947) was the first to speak of the structure associated with the chromatin as a "hollow spindle."

SUBRAMANIAM'S CHROMOSOMES. SUBRAMANIAM (1948) supports the view that the chromosomes are few, large and



Text-Figure 3.

The drawings marked 1, 2 and 3 are copies of LIETZ's (1951) Abb. 1c. The drawing to the left is a three-dimensional interpretation of his figures, indicating that "1" is a median optical section, "3" an upper optical section, and "2" a section between "1" and "3". If LIETZ's "3" had been drawn critically, the periphery would have been hazy and out-of-focus.

deeply stained by the Feulgen procedure, but the small haploid numbers which he has inferred are refuted by genetical analysis. If the units he considers to be haploid genomes have been properly designated, they must be aggregates of larger numbers of chromosomes. His procedure is adequately described but is not one which has been used by other yeast cytologists, since the fixation involves treatment with ammonia. It is possible that the dark structures he photographed may not be Feulgen-positive, because they are very dark and large and the Feulgen procedure has not yielded similar results in other hands. Since SUBRAMANIAM's photographs often show highly refractile clear centers in the darkly stained granules, it is possible that they represent highly refractile Feulgen-negative granules which have been photographed with a small numerical aperture. This procedure can produce red granules of high density

because of light reflected from the red background provided by residual fuchsin in the cell.

During a visit to Dr GERSH's laboratory, he demonstrated to Drs LINDEGREN and MUNDKUR the possibility of obtaining a false red image by reducing the aperture when inspecting Feulgen preparations of yeast cells. The preparations described by RAFALKO, LINDEGREN and RAFALKO, and MUNDKUR were examined by full aperture and found to be red because of intrinsic color (rather than color imparted from the background). The senior writer has personally examined Dr LEVAN's preparations with full aperture and can testify that the redness of the chromosomes is intrinsic.

Reconciliation of different points of view. In view of the findings reported herewith, it seems possible to reconcile the differences between the WAGER-LINDEGREN-RAFALKO view that the vacuole with its associated organelles is the nucleus and the view held by GUILLIERMOND (1910), FUHRMANN (1906), KATER (1927), SINOTO and YUASA (1941), LEVAN (1947), RENAUD (1938), DELAMATER (1950), LIETZ (1951) and many others that the nucleus is a smaller body attached to the vacuole. The Carnoy-perchloric-Giemsa procedure in combination with data on DNA synthesis (OGUR, MINCKLER and McCLARY, 1953) and X-ray killing (SARACHEK, 1954) indicates that the chromosomes divide either before the first bud appears or shortly thereafter. This inference is drawn from the fact that the cell contains the double amount of DNA and 2 x-ray targets at this time.

LINDEGREN and LINDEGREN (1946) and LINDEGREN (1949) observed large paired basophilic intravacuolar structures which appeared to split longitudinally and inferred that the chromosomes lined up end-to-end and that mitosis occurred inside the vacuole. These structures were studied in detail by TOWNSEND and LINDEGREN (1953) and found to appear in cells containing well-formed crescents after growth under anaerobic conditions. They were best demonstrated with toluidine blue at high pH and it was suggested that their peculiar shape might be due to solution and recrystallization either of metaphosphate or of chromatin or both. The fact that chromatin may break away from its normal polarized position and appear free in the vacuole (Fig. 14) suggests one manner in which this might occur. The crystalloids do not fit into the scheme (presented in Text-figures 1 and 2) developed from the present study of cells undergoing progressive normal growth, stained with

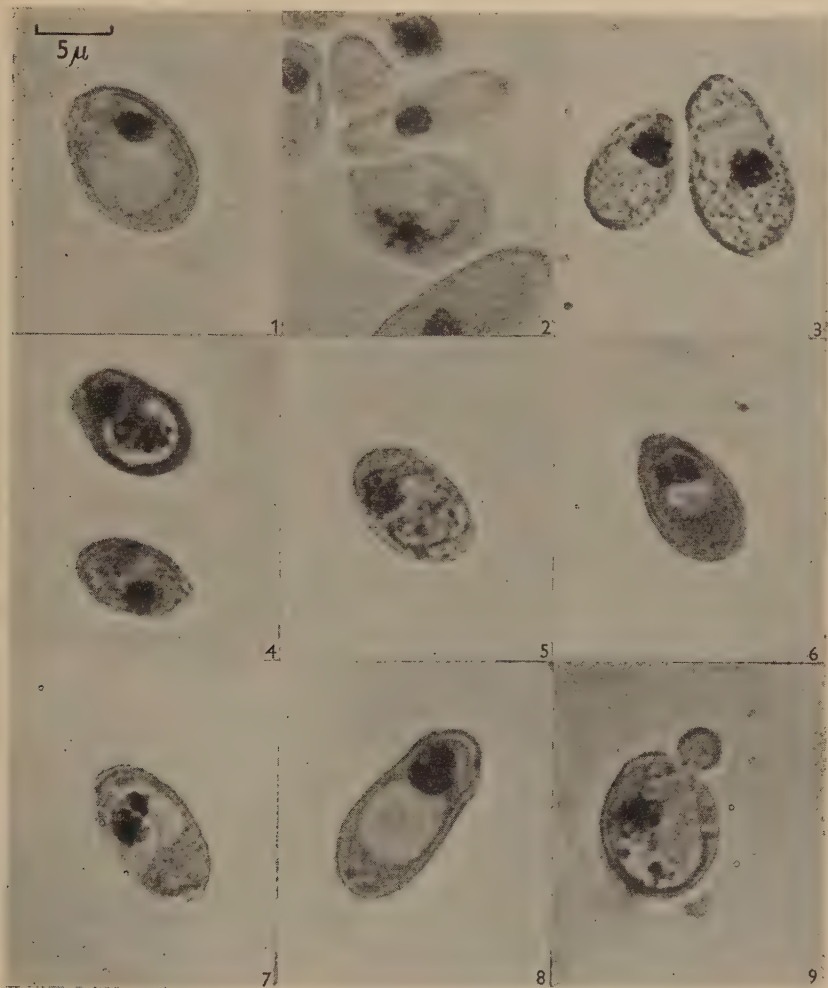
Giemsa, after basophilic material other than chromatin has been removed from the cell. The intravacuolar crystalloids are most conspicuous in dormant cells when the chromosomes are retracted on the spindle. The view of GUILLIERMOND (1910) that some of the crystalloidal substances observed in the vacuole are "metachromatin" reserves is accepted, although his contention that chromatin does not occur in the vacuole is refuted by Figures 11, 12 and 13 which show chromatin in the vacuoles of cells free of "metachromatin" (metaphosphate?) after staining with Giemsa. His view that the division is amitotic is negated by the genetical evidence.

SINOTO and YUASA, LEVAN, LINDEGREN and RAFALCO, LIETZ and others have shown telophases at a stage when all the chromatin is on the spindle and the vacuole is empty, making agreement on the view that mitosis occurs on the spindle practically unanimous.

Disagreement centers chiefly about whether or not chromatin normally occurs in the vacuole. RAFALCO's critical study (1946) which first revealed Feulgen-positive strands in the vacuole should have established this fact but the numerous papers which either do not quote him or state otherwise show that this is not so. Authors who insist that chromatin does not occur in the vacuole often show granules of chromatin in the vacuole which they describe as artifacts. LEVAN drew numerous cells containing chromatin in the vacuole but described it as an abnormality produced as the result of C-mitoses, but C-mitoses do not cause the extrusion of chromatin from the nucleus. It may be inferred, therefore, that if chromatin appears in the vacuole as a result of C-mitoses, this supports the view that chromatin normally is present in the vacuole. SINOTO and YUASA also showed chromatin in the vacuole but described the spindle with its associated chromosomes as the nucleus. Carnoy-perchloric-Giemsa preparations show that chromatin appears in the vacuole connected by long threads to the chromatin on the spindle shortly after mitosis has occurred on the spindle. These photographs confirm the presence of chromatin in the vacuole reported by J. S. RAFALCO and by LINDEGREN and M. M. RAFALCO.

Is the spindle intra- or extra-vacuolar? LINDEGREN and RAFALCO found preparations showing the spindle to be intravacuolar. This view might have been reached by inference, in the absence of direct evidence, since it is in general agreement with the occurrence of intravacuolar spindles in the Ascomycetes. It explains the facility

with which the chromatin on the spindle extends into the vacuole shortly after budding.

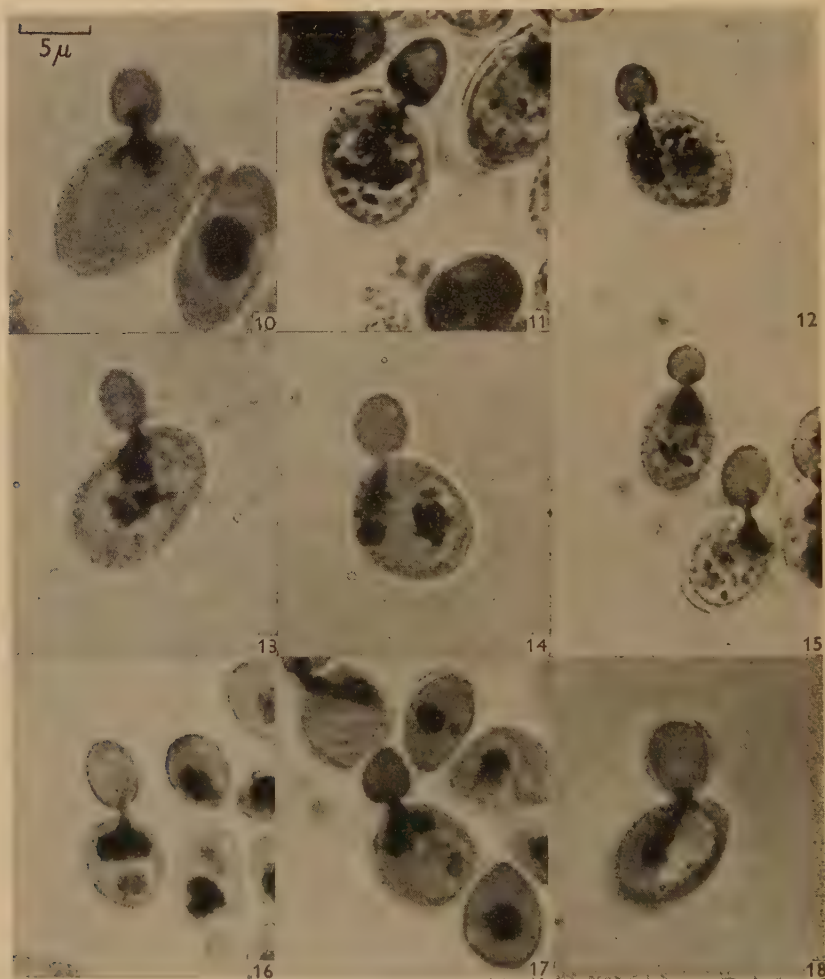


Figures 1 to 9.

Photographs of wet mounts of yeast cells prepared by the Carnoy-perchloric-Giemsa procedure. See discussion in text.

An answer to the criticism of LIETZ. LIETZ (1951) following an extensive investigation of the yeast nucleus, made an important contribution by summarizing his views and WINGE's in clear and unambiguous language. The different points

are numbered in the following translation so that each one may be answered specifically.

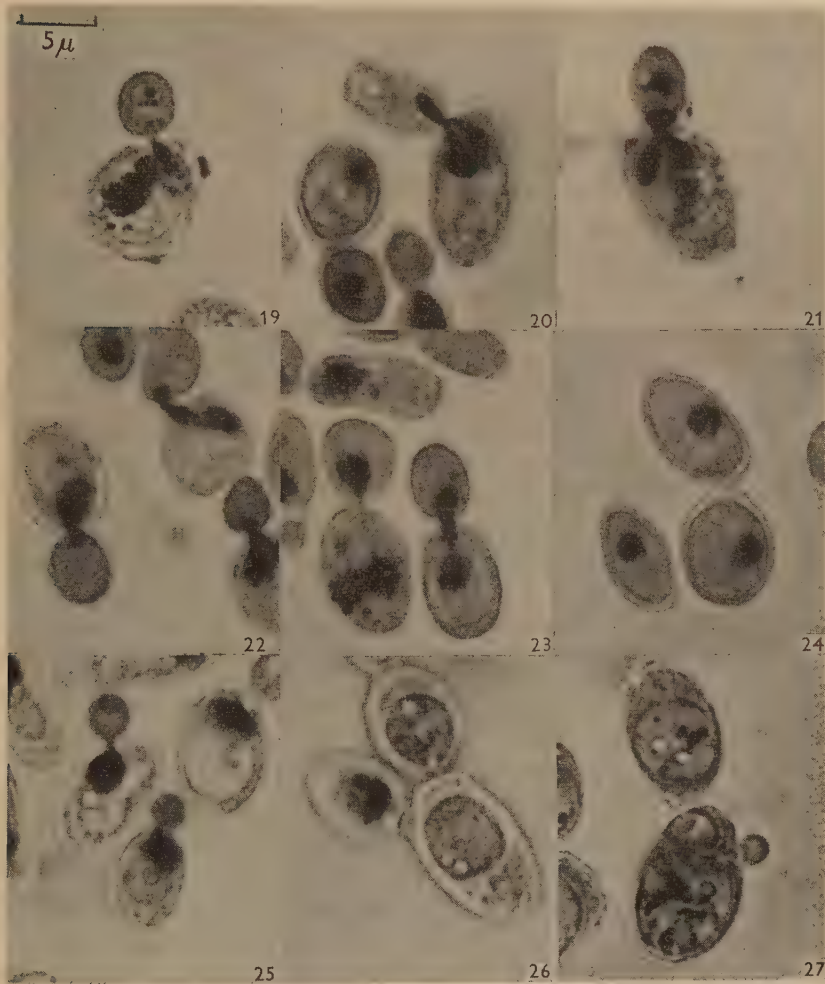


Figures 10 to 18.

Photographs of wet mounts of yeast cells prepared by the Carnoy-perchloric-Giemsa procedure. See discussion in text.

“Even without considering the fact that (1) I have been unable to demonstrate any nuclear positive elements in the cell vacuole, I cannot agree with LINDEGREN’s interpretation because the following questions immediately arise: (2) Which of the vacuoles is the

nucleus if the cell contains more than one vacuole? (3) Where is the nucleus if the yeast cell, as is often the case in young cultures, contains no vacuole? (4) In starving cells in which the vacuole almost



Figures 19 to 25.

Photographs of wet mounts of yeast cells prepared by the Carnoy-perchloric-Giemsa procedure. See discussion in text.

Figures 26 and 27.

Photographs of cells fixed in Carnoy and stained with Lindegren's volutin stain. See discussion in text.

completely fills the cell space, why is the so-called nucleus disproportionately large while in similar cells in good nutrition it is considerably smaller? (5) The structure called the centrosome (spindle) by LINDEGREN gives a Feulgen-positive reaction in every case. (6) Although LINDEGREN is correct when he states that the centrosome is a cell structure whose biological function is more important than its chemical composition, it is not comprehensible why the Feulgen-positive cell structure whose relation to cell budding throughout corresponds to that of a nucleus should be considered the centrosome while a Feulgen-negative continually variable structure, the vacuole, should be considered the nucleus. (7) It is singular that the division of LINDEGREN's centrosome should occur after the nuclear division. (8) My opinion agrees completely with that of WINGE (1948, page 311): 'The so-called chromosomes observed by LINDEGREN (1945b), which are vitally stained with methylene blue or toluidene blue and which participate in Brownian movement in the nuclear sap, cannot actually be the chromosomes' especially since I have never been able to observe the same picture as that described by LINDEGREN with toluidine blue. (9) LINDEGREN's concept concerning the appearance of his centrosome can be interpreted, however, in another sense to establish the nuclear membrane, the nuclear plasma and the half-moon shaped chromosomal substance. (10) In conclusion, therefore, one may say that the observations and the results of most investigators are in essential agreement. It has been found that the yeast nucleus differs from the nucleus of higher plants in the following points: (11) a. Through the characteristic position of the chromosome substance. (12) b. Through the absence of typical stainability with the common nuclear stains. (13) c. Through the absence of a nucleolus inside the nucleus. (14) According to this interpretation the volutin granules are homologues of the nucleolus."

The following answers deal with the respective items numbered above:

(1) LEVAN has pointed out that staining of the yeast cell is "whimsical". RAFALKO's findings of Feulgen-positive material in the vacuole are not negated by the failure of LIETZ to achieve a similar result, especially since the present paper shows that the occurrence of chromatin in the vacuole depends upon the stage in the growth cycle at which the cells are fixed.

(2) The vacuole in the yeast cell may be lobed, but each cell

contains only a single vacuole. Each of these lobes connects with the spindle, and, since the spindle is intravacuolar, the connection encloses the spindle. The most common observation concerning "multiple" vacuoles is in an elongated yeast cell in which the spindle lies in the center with apparently two vacuoles — one attached to each side. It is inferred that only a single vacuole is present which encloses the spindle in a central position.

(3) Although unstained cells in young cultures often appear to have no vacuole, one vacuole is always present in each cell and can be revealed by staining with dilute Lugol's solution. The single vacuole in many young cells is concealed in the unstained cell by a relatively homogeneous penetration of the cytoplasm by glycogen. The vacuole is destroyed by fixation procedures such as drying the cell, heat fixation and aceto-carmin stain.

(4) Yeast cells vary extraordinarily in size. It is not unusual to find variations of volume of one-hundred fold in a single culture. The vacuole is not the nucleus; it is merely the nuclear vacuole, *i.e.*, the membrane surrounding the nucleus and the contained hyaloplasm. Variations in size result from enlargement of the vacuole by increasing the content of nuclear sap. A large vacuole does not mean more chromatin but merely more nucleoplasm and does not imply differences in amounts of chromosomal or genic materials.

(5) The structure which LINDEGREN formerly called the "centrosome" is now identified as the spindle (TOWNSEND and LINDEGREN, 1953). It is acidophilic, Feulgen-negative (not Feulgen-positive) and solid, associated with an external cap of chromatin. The statement by LIETZ is a mis-quotation.

(6) The nucleus, according to the concept presented herewith, in addition to the Feulgen-positive chromosomes, comprises the Feulgen-negative spindle and the (Feulgen-negative) vacuole (considering the nuclear membrane and hyaloplasm as the vacuole). The chromosomes are attached to the outside of the spindle (which lies in the vacuole) and extend into the nuclear sap which fills the vacuole.

(7) The division of the spindle (centrosome) occurs after the nuclear division, because, like most solid spindles, the separation of the chromosomes at anaphase is facilitated by elongation of the spindle.

(8) J. S. RAFALCO, and LINDEGREN and M. M. RAFALCO described Feulgen-positive structures in the vacuole. The present paper

points out that during some stages of the growth cycle the vacuole is filled with chromosomes while at other stages the chromosomes are retracted on the spindle. The chromosomes in the vacuole are identified by perchloric extraction which removes basophilic material other than chromatin after which the chromatin is stained by Giemsa. These data have proved that chromatin occurs in the vacuole. GUILLIERMOND's view that basophilic reserves occur in the vacuole is also accepted and the view previously proposed by LINDEGREN that mitosis occurs in the vacuole is rejected. Some of the basophilic material found in the vacuole and originally identified by LINDEGREN as chromosomal undoubtedly belongs to the category of basophilic reserves, since these earlier observations were not controlled by the removal of basophilic material other than chromatin from the cell.

(9) LIETZ is proposing an homology between a solid (the spindle) and a fluid (the hyaloplasm), and is placing external chromatin inside a solid structure.

(10) The yeast nucleus is not homologous with the nucleus of higher plants but resembles the nuclei of other ascomycetes in every point:

(11) a. Through the characteristic polarization of the chromosomes which are oriented at all times toward the spindle (which LIETZ has called the "nucleus").

(12) b. Through the high RNA/DNA ratio which makes staining with the common nuclear stains difficult before extraction with perchloric acid.

(13) c. Through the presence of a nucleolus in the nuclear vacuole (LIETZ apparently did not grow cells rapidly enough to produce the nucleolus).

(14) An extensive study of the position of the volutin in the yeast cell has shown that it occurs only inside the nuclear vacuole (LINDEGREN, McCLARY and WILLIAMS, 1955). By "volutin granules" LIETZ probably means the mitochondria which lie in the cytoplasm. It is not necessary to suppose them to be homologues of the nucleolus since a true nucleolus is demonstrable inside the nuclear vacuole.

An obvious relation between bacterial and yeast cytology. ROBINOW (1953) has pointed out that the spores of *Bacillus megaterium* go through a transformation from

resting spore to dividing cell similar to that which has been reported herewith in the yeast cell. His figure 1 (p. 380) shows a central vacuole with an attached half-moon of chromatin. Optical sections through this structure suggest that it is almost identical to the spindle of the yeast cell coated with a layer of chromatin. Sixty minutes after transfer of the spore to nutrient agar, the chromatin is spun out into chromosomes which occupy the center of the cell. The similarity between the bacterial preparations and those from yeast cells is so striking that it may be assumed that the process is fundamentally the same. The view that the chromatin is layered on a solid spindle suggests that the extrusion of chromatin from the spores by certain methods of fixation may be due to the extrusion of the solid, chromatin-coated spindle during the shrinkage of the spore.

Summary and conclusions.

A critical examination of well fixed yeast cells killed at different stages in the growth cycle shows that the distribution of chromatin in the yeast cell varies depending on the stage in the growth cycle. Our data support the view (1) that the spindle is intravacuolar, (2) that mitosis occurs on the spindle, (3) that the chromosomes spin out after mitosis to extend into the vacuole, (4) that a large nucleolus appears in the nuclear vacuole of rapidly growing well-nourished cells, and (5) that in the presence of adequate amounts of phosphate and otherwise favorable conditions the chromosomes are covered with metaphosphate.

Acknowledgment.

We are indebted to Dr MAURICE OGUR for his assistance and advice in the use of perchloric acid for the extraction of RNA and metaphosphate. Our staining procedure derives its value from this differential extraction and is the method of choice for following chromatin in the yeast cell.

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ACETONE-DRIED TYPHOID BACTERIA AS A STABLE ANTIGEN FOR THE STANDARDISED Vi AGGLUTINATION TEST ¹⁾

by

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(Received April 5, 1955).

The value of the Vi agglutination test as a tool for the detection of the chronic carrier first noted by FELIX, KRIKORIAN and REITLER (1935) is established by the work of various authors. The standardization of the test has been advocated by FELIX (1950). Technical difficulties present themselves when the suspensions are to be prepared. There is some difficulty in obtaining a subculture of the Vi I strain (BHATNAGAR *et al.*, 1938) which yields a suspension that is stable in that it shows negative reactions with normal sera. Moreover, the stability of the formalinised suspension tends to deteriorate when it is stored at 4°C. Since acetone-dried bacteria have been successfully employed first for the preparation in the horse of therapeutic antityphoid Vi + O serum (HENDERSON, AMIES and STEABEN, 1940) and afterwards as typhoid vaccines (RAINSFORD, 1942; GIOVANARDI and MONACI, 1943; LANDY, 1953), it was thought that this method might prove suitable for the preparation of a stable antigen for the Vi agglutination test.

Material and methods.

The strain Vi I (BHATNAGAR) was plated and six of the best smooth colonies were subcultured in broth and on agar. Agglutination tests with ten "normal" sera were performed and Roux bottles were inoculated with the subculture which gave negative reactions with these sera. After 18 hours incubation the bacterial output was dried in acetone according to the procedure described by LANDY (1953) as follows: The bacterial growth from each bottle is suspended in

¹⁾ We thank Dr A. FELIX for the revision of this paper.

10 ml distilled water. One volume of this heavy suspension is poured out in three volumes acetone. After two hours at room temperature the bacterial mass is sufficiently settled. The yellow coloured supernatant fluid is decanted. The bacteria are washed twice with acetone. Finally the bacteria are resuspended in acetone and kept 24 hours at 37°C. in order to sterilize the suspension. The acetone is sucked off through a glassfilter 26—G3 Jena and the bacterial mass is dried in vacuo over P_2O_5 . The preparation obtained is powdered in a ball mill and mixed with NaCl p.a., also powdered in the ball mill (24 parts of bacterial powder are mixed with 425 parts NaCl by weight). The mixture is again treated in the ball mill. Immediately before use 900 mg of the powder is suspended in 100 ml distilled water, giving a suspension of about 2000 millions of bacteria/ml in physiological saline.

Results.

After storage of the dried bacteria for nine months at room temperature suspensions were prepared and examined with the Standard TVi Serum of FELIX and BENSTED (1954). In accordance with the requirement agglutination reactions readable with the naked eye were recorded up to a dilution of 1/1400. The suspension was compared with a fresh Vi suspension obtained from the Central Public Health Laboratory, Colindale (London) in the examination of eight other sera. Seven of these sera gave the same titres with both suspensions (differences less than one dilution). The eighth serum gave a $2\frac{1}{2}$ -fold titre with our suspension (1/100 : 1/40), repeatedly. The readings with the dilutions of the Standard Serum were for both suspensions the same.

The suspension was also tested with "normal" sera. In the first series of fifty sera the suspension showed sometimes traces of agglutination in the lowest dilutions, resp. $1 \times$ in a dilution of 1/20, $2 \times$ 1/10 and $4 \times$ 1/5. These traces of agglutination could hardly be interpreted as positive reactions. In a further series of hundred sera half the number showed the same phenomenon. The appearance of these trace reactions was, however, entirely different from the total clarification shown by positive sera of typhoid patients or carriers.

The results after storage for nine months were similar to those obtained with the suspension immediately after its preparation.

Summary and conclusions.

A technical improvement is described of the typhoid Vi agglutination test in which acetone-dried suspensions are used.

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DETERMINATION OF THE Vi-ANTIGEN CONTENT OF ACETONE-DRIED TYPHOID VACCINES

by

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(Received April 9, 1955).

INTRODUCTION.

In many countries the heat-killed phenolized typhoid vaccine has now been used for half a century. Mostly it is administered in a series of three injections (0.5, 1, and 1 ml of a bacterial suspension of 1000 millions bacteria per ml) each time with an interval of one week.

In 1934 FELIX and PITT (1934) discovered the Vi-antigen. BENSTED (1940) and FELIX (1951) observed a high incidence of typhoid Vi-strains isolated from the infections of human beings. However the heat-killed phenolized vaccine failed to stimulate circulating Vi-antibody in rabbits and in man. Therefore FELIX (1951) considered the phenolized vaccine as being defective in antigenic composition.

Although the relation of the Vi-antigen to the pathogenicity of the typhoid bacteria in man is not quite clear and conversely little is known of the protective value of the anamnestic existence of Vi-antibody in typhoid fever patients, still at least some value must be attached to a vaccine capable of producing Vi-antibodies.

FELIX *et al.* (1941a, 1941b) approached this problem by preparing an alcoholized vaccine in which the Vi-antigen is preserved during periods of years, provided that the vaccine is kept at 4°C.

However, the rapid deterioration of the Vi-antigen in this vaccine at room temperature is a serious impediment (RAINSFORD, 1942). LANDY (1953) prepared an acetone-killed and -dried vaccine which

¹⁾ We thank Dr M. LANDY for the revision of this paper.

contains Vi-antigen and is stable at room temperature. These investigators followed the method of HENDERSON *et al.* (1940). By this method a dried talc-like bacterial powder is obtained. This product is mixed with dry powdered chemically pure sterile NaCl in amounts required to yield a saline solution when rehydrated with distilled water. The vaccine disperses easily with a minimum of shaking. Microscopic examination reveals a uniform suspension of bacteria with no evidence of clumped organisms.

The heat-killed phenolized vaccine is known to cause unpleasant general and local reactions after inoculation. If it becomes feasible to standardize the Vi- and O-antigen content of the stable acetone vaccine, prepared from selected strains, the greater immunogenic potency of this product may permit to reduce the vaccine dose without loss of immunizing effect. In this manner undesirable reactions might be reduced in incidence and severity.

This paper reports results of the determinations of the Vi-antigen content of several strains of typhoid bacteria used in vaccine production. Determinations were carried out with the quantitative complement fixation and erythrocyte sensibilization reactions.

Our results show that with the aid of these methods serological determination of the Vi-antigen content of acetone-dried typhoid vaccines is possible.

MATERIALS AND METHODS.

1. Preparation of crude Vi-antigens.

The Vi-antigen was derived from acetone-dried typhoid strains and coli strain 5396/38¹⁾ by the process described by LANDY (1953) and WEBSTER *et al.* (1952). The typhoid strains were taken from the lyophilized state and cultivated on veal infusion agar plates (18 hours at 37°C.). A smooth colony which was strongly agglutinated by Vi-serum was selected to seed a veal infusion broth culture (18 hours at 37°C). Veal infusion agar in Roux-bottles were seeded with the broth culture. We could confirm the communication of LANDY (1952) that the coli strain tends to a rapid dissociation into opalescent colonies (Vi-containing) and translucent colonies (free from Vi-antigen). When the strain is cultivated on serum agar this

¹⁾ Dr LANDY kindly sent us a lyophilized culture of the Vi-strain of *E. coli* 5396/38.

dissociation is not so rapidly observed. Therefore the coli strain is cultivated from the lyophilized state on 30 per cent serum agar plates (incubation 18 hours at 25°C). After repeated colony selection the coli strain was cultivated in one or two Roux-bottles with veal infusion 5 per cent serum agar (incubation 18 hours at 25°). Growth was harvested in veal infusion broth and this was used to seed 5 per cent serum veal infusion agar in Roux-bottles, which were incubated at 25°C for 18 hours.

The growth in each flask was harvested in 10 ml distilled water. One volume of the harvest was poured into three volumes of acetone. After a few hours' standing at room temperature the flocculated bacterial mass had settled down and the acetone-water mixture was carefully decanted.

The organisms were washed once with acetone, resuspended in acetone and killed by incubating at 37°C. during 24 hours. Finally the acetone was removed by filtration through a G₃ glass filter and the residual bacterial mass was dried in vacuo over P₂O₅. The dried bacterial mass was ground in a ball mill to a fine talc-like powder.

To free the Vi-antigen one gram portions of dry powder were shaken with 100 ml volumes of saline during half an hour. The suspensions were centrifuged at 5000 G in an angle centrifuge for 30 minutes. The supernatant fluid (which contains more than 95% of the Vi-antigen as measured by means of the erythrocyte sensitization reaction) was carefully decanted and 1/100 volume of 1 per cent merthiolate was added. The antigens can be stored without deterioration at 4°C. for some months.

2. Preparation of Vi-antisera.

Groups of 10 rabbits were immunized with living cultures of *P. ballerup*. The bacteria were given in amounts of 1000 millions in one series of three weekly intravenous injections. Six days after the last injections the animals were bled by heart puncture. After pooling the sera 1/100 volume of 1 per cent merthiolate was added and the sera were kept at 4°C. in the refrigerator.

3. Quantitative complement fixation reaction.

This reaction was carried out with three 50% units (Ko) of complement (COHEN, 1953). Fixation was performed at 4°C. for 18

hours. The hemolytic system was prepared by sensitizing a 1 per cent (spectrophotometrically standardized) suspension of sheep red blood cells with optimal amounts of hemolytic antibody. Antigen antibody and complement were added in quantities of 0.4 ml. The total volume of the reacting mixture after the addition of the hemolytic system was 2 ml. Incubation for lysis took place at 37°C. for 15 minutes. Thereafter the tubes were centrifuged and the percentage hemolysis was determined spectrophotometrically and rounded off to units of 5 per cent.

Sheep-blood was preserved in a modified Alsever solution. The same batch of complement conserved chemically according to RICHARDSON (COHEN, 1953) was used during the experiment.

4. Erythrocyte sensibilization reaction (SPAUN, 1951).

Two volumes of a spectrophotometrically standardized 1 per cent suspension of sheep red blood cells were sensitized with three volumes of the antigen dilution under investigation in a waterbath at 37°C. for 15 minutes. Washing the sensitized cells is unnecessary as absorption of the Vi-antigen is complete. To perform the test 0.5 ml of sensitized cells are added to 0.5 ml of serum dilution. All the dilutions are made in 0.85 per cent saline with 0.1 per cent gelatin. Results are read after an incubation at 4°C. during 18 hours.

RESULTS.

Experiment 1a: Complement fixation reaction.

Table 1 illustrates the reaction of various dilutions of the coli Vi-antigen in serial dilutions of a Vi-antiserum in a chessboard test.

Results are given in percentages of hemolysis rounded off to units of 5.

From the results mentioned in table 1 it is clear that neither the antigen dilutions nor the serum dilutions exhibit important anti-complementary effects (see controls with 1 and 2 Ko units of complement). Distinct inhibition of the reaction exists in the antigen excess zone. It is not altogether clear whether the optimal antigen antibody ratio is constant. This value varies between $1024/300 = 3.5$ and $8196/1012 = 8$. Perhaps this variation may be ascribed to experimental errors.

From our results it can be concluded that it is possible to determine the Vi-antigen content of different strains by titrating several

TABLE 1.

Results of a chessboard experiment of a Vi-antigen titration (coli 5396/38) in the complement fixation reaction. The figures represent percentages of hemolysis.

Serum dilution	Antigen Dilution								Controls	
	1/256	1/512	1/1024	1/2048	1/4096	1/8192	1/16384	1/32768	1 Ko	2 Ko
1/200	0	0	0	0	0	5	80	100	35	100
1/300	10	5	5	10	10	10	85	100	—	—
1/450	45	35	15	5	15	15	85	100	—	—
1/675	90	75	50	40	15	20	85	100	—	—
1/1012	100	90	95	90	90	85	95	100	—	—
1/1518	100	100	100	100	100	100	100	100	—	—
Controls										
1 Ko	35	—	—	—	—	—	—	—		
2 Ko	100	—	—	—	—	—	—	—		

dilutions in a complement fixation test against a constant dilution of serum. The unit of antigen is the dilution which gives maximal complement fixation with this amount of serum.

Experiment 1b:

Table 2 gives a typical result of a titration of the same coli antigen with the erythrocyte sensibilization reaction in a chessboard test.

TABLE 2.

Results of a chessboard experiment of a Vi-antigen titration (coli 5396/38) in the erythrocyte sensibilization test.

Serum dilution	Antigen dilution								
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800
1/250	+++ ¹⁾	+++	+++	+++	++	+	+	±	—
1/500	+++	+++	+++	+++	++	+	+	±	—
1/1000	++	++	++	++	++	+	+	±	—
1/2000	+	±	±	±	±	—	—	—	—
1/4000	—	—	—	—	—	—	—	—	—

¹⁾ +++ = complete agglutination.

An inhibition of the reaction in the antigen excess zone has not been observed at least in the dilution scale which was used during

the experiment ¹⁾. Following the directions of LANDY we decided to accept as antigen titer the highest dilution giving a complete reaction.

Experiment 2:

Two batches of acetone-dried powders were prepared independently on separate days from the following strains: 8 typhoid strains used for vaccine production in this Institute, and the strains Ty 2, O 901, H 901, coli 5396/38. The method described by SCHOLTENS *et al.* (1956) was used. These two batches were labelled A and B respectively. All the bacterial extracts were analyzed for Vi-antigen content in the complement fixation test (serum dilution 1/625) and erythrocyte sensibilization test (serum dilution 1/500). All the reactions were performed on the same day. In table 3 results are given. If in the complement fixation test two successive antigen dilutions gave an optimal reaction, the geometrical mean was considered as the titer.

TABLE 3.

Reciprocal values of titers of Vi-antigen content in 12 bacterial strains in the complement fixation and erythrocyte sensibilization test.

No.	Strain	Erythrocyte sensibilization test		Complement fixation test		Mean erythrocyte sensibilization test	Mean complement fixation
		A	B	A	B		
1	643 ¹⁾	200	200	1024	1024	200	1024
2	2281	100	100	183	366	100	259
3	2342	100	100	366	366	100	366
4	2673	50	50	366	366	50	366
5	2750	100	200	366	366	142	366
6	2794	100	100	512	1024	100	724
7	15310	100	100	512	366	100	433
8	16148	200	100	512	366	142	433
9	Ty 2	50	25	256	183	35	216
10	coli ²⁾	800		4096		800	4096
11	O 901	<25 ³⁾		<16 ³⁾			
12	H 901	<25 ³⁾		<16 ³⁾			

¹⁾ Bandung

³⁾ Lowest dilution tested

²⁾ 5396/38

¹⁾ Inhibition may be observed when large amounts of antigen are used as sensitizing agent.

In columns 5 and 6 of table 3 the geometrical means are given of the duplo values A and B in both reactions.

From the results in table 3 it is clear that, generally speaking, good agreement exists between the double values A and B in each strain. It seems to be possible to distinguish serologically the strains with much antigen (e.g. no. 1 and 10) and the strains with less antigen (e.g. no. 9). In the dilutions tested the control strains H 901 and O 901 (without Vi) gave completely negative reactions.

DISCUSSION.

In the introduction of this paper our provisional aim was stated to be the production of a typhoid vaccine in which the two most important antigens Vi and O are retained without special means of preservation in amounts as high and constant as possible. The investigations of LANDY (1953) made it clear that these conditions may be fulfilled by using an acetone-killed and -dried bacterial vaccine. Our own investigations have confirmed the communications of LANDY. Moreover it was possible to titrate crude solutions of Vi-antigen in the complement fixation test and erythrocyte sensibilation test. LANDY *et al.* (1954) analysed purified Vi-antigen from different sources serologically with these reactions and the quantitative precipitation test. We performed the latter test also with our crude extracts. As was to be expected (the antiserum in this test is used undiluted and the antigen is relatively concentrated), our results were obscured by other antigen-antibody reactions occurring simultaneously. However, the complement fixation test and erythrocyte sensibilation test are carried out with highly diluted reagents. The tests could always be read without any difficulty and gave clear-cut results. Moreover, control antigens from strains in the W-phase, H 901 and O 901 gave completely negative results with all the serum dilutions used. So we may safely assume that our results are specific and give a good impression of the Vi-antigen content of our strains. As in the reactions with pure antigens (LANDY *et al.*, 1954) there exists a reasonable positive correlation between mean titers in complement fixation and erythrocyte sensibilation tests (Rank correlation coefficient 0.758; critical value 1% level at $N = 10$: 0.746). The Bandung strain (643) seems to have a high Vi-antigen content. Results with the Ty 2 strain have been rather disappointing. We can confirm the communication of LANDY (1952) that the coli strain 5396/38 has a very high Vi-antigen content. The

object we had in view was favourably affected by the fact that the two bacterial powders derived from one strain, but independently prepared under the same conditions, always had the same or nearly the same titer.

It is also possible to standardize the Vi-antigen content of acetone-dried typhoid vaccine serologically. As the serological reactions showed some differences when carried out on different dates, we now used the dried coli powder as a standard. Of course we are not sure now that two strains with the same Vi-antigen content are indeed capable of producing the same amount of antibody. There may exist functional deficiencies in antigenic capacity. LANDY *et al.* (1954) found a serological and antigenic deficiency in a purified antigen derived from the Ty 2 strain. However, differences found by these investigators may be accounted for by the purification process. Investigations will be carried out in this laboratory to compare the antigenic stimulation in animals of our strains with known antigen content.

S u m m a r y.

A number of typhoid strains and coli strain 5396/38 are compared for their Vi-antigen content with the aid of complement fixation and erythrocyte sensibilization tests. Acetone-killed and dried germs were used as source material. Parallel batches derived from the same strain showed good agreement. Moreover, results with erythrocyte sensibilization and complement fixation reaction were closely correlated. The conclusion is drawn that serological determination of the Vi-antigen of acetone-dried typhoid vaccine is possible. Further investigations, however, must be carried out to compare the functional capacity to produce antibodies of strains with the same serologically determined content of Vi-antigen.

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SUR CERTAINES ALTÉRATIONS DE LA MULTIPLICATION DES BACTÉRIES SENSIBLES PROVOQUÉES PAR LA STREPTOMYCINE

par

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(Reçu le 8 mai 1955).

Nous avons utilisé précédemment le turbidimètre photo-électrique pour examiner quelques aspects de l'action de la streptomycine sur le développement des bactéries sensibles (4,7) ou résistantes (8) à son effet bactériostatique. Le turbidimètre permet, plus commodément que d'autres moyens d'investigation, d'analyser le comportement des bactéries pendant la période même où l'antibiotique les influence, et leur comportement dans les subcultures suivantes. Une telle étude entraîne des comparaisons qui constitueront le principal objet du présent mémoire.

TECHNIQUE.

Les techniques utilisées sont les mêmes que précédemment (4, 7, 8) et il suffira de les rappeler brièvement.

Des tubes spéciaux en verre, optiquement vides, reçoivent successivement 8 ml de bouillon nutritif riche (Brain heart infusion Difco), 1 ml de solution de streptomycine dans le même bouillon, et 1 ml de cultures en bouillon de *Staphylococcus pyogenes* ou d' *Escherichia coli*, cultures âgées de 20 à 24 heures et diluées en bouillon frais, s'il y a lieu. Dans le cas de *Mycobacterium tuberculosis*, le bouillon est remplacé par le milieu liquide de Dubos au "Tween 80" et à l'albumine, et la semence est fournie par une culture dans le même milieu âgée de 7 jours.

Immédiatement après leur préparation (temps zéro), les tubes sont placés dans le turbidimètre pour une première mesure. L'appareil est réglé, pour chaque tube individuellement, de sorte que

le tambour marque 100; les repères sont notés et l'appareil sera réglé à nouveau de la même manière, chaque fois que la même culture sera examinée. Les valeurs trouvées ne seront que relatives, mais on pourra les comparer entre elles, pourvu qu'il s'agisse du même tube.

La première mesure finie (temps zéro), les tubes sont portés sans retard dans un bain-marie à 37° C. (*staphylocoque*, *coli*) ou à 38° C. (*bacille tuberculeux*). Périodiquement, ils sont retirés du bain-marie, fortement agités et soumis à une nouvelle mesure, puis reportés au bain-marie.

Les lectures au tambour dépendent de la quantité de lumière transmise à travers les tubes. Les chiffres sont donc d'autant plus grand que le liquide est plus limpide, et inversement. Ce sont ces chiffres exclusivement qu'on trouvera dans nos tableaux et graphiques.

Nous nous sommes servis de plusieurs souches de *Staph. pyogenes*, d'*E. coli* et de *Myc. tuberculosis*. Toutes les souches d'une même espèce donnent des résultats semblables. Aussi nous contenterons-nous de citer ceux obtenus avec *Staph. pyogenes* souche Oxford, *E. coli* souche K 12 et *Myc. tuberculosis* souche B.C.G. de l'Institut Pasteur de Bruxelles. Les concentrations minimum inhibitrices de la streptomycine pour ces souches sont les suivantes (pour un ensemencement de 10⁷ bactéries par ml):

Staph. pyogenes (en bouillon coeur-cerveau): 125 µg/ml

E. coli (en bouillon coeur-cerveau): 31 µg/ml

Myc. tuberculosis (en milieu liquide de Dubos): 0.1 à 0.2 µg/ml

Rappelons-nous que, pour mesurer plus aisément le développement du trouble de *Staph. pyogenes* et d'*E. coli*, nous nous plaçons dans des conditions où la streptomycine perd beaucoup de son activité: le bouillon est très riche en facteurs antagonistes de l'antibiotique; l'ensemencement est massif. Dans des bouillons moins riches et ensemencés moins abondamment, les concentrations bactériostatiques de streptomycine sont beaucoup plus faibles (de l'ordre de 0.1 à 1 µg/ml).

Les concentrations de streptomycine habituellement utilisées ont été inférieures aux concentrations bactériostatiques, mais proches de celles-ci. Elles ont été, pour un ensemencement d'environ 10⁷ cellules par ml:

pour *Staph. pyogenes*: 100 µg/ml

pour *E. coli*: 10 µg/ml

pour *Myc. tuberculosis*: 0.01 µg/ml

RÉSULTATS.

Les tableaux 1, 2 et 3 montrent que le développement des germes de chaque espèce microbienne est remarquablement constant lorsque les conditions de culture sont maintenues constantes. Dans les trois tableaux, les tubes n^{os} 1 et 2 ont été choisis, parmi 10 tubes semblables ensemencés en même temps, parce que, de tous, ils suivaient

TABLEAU 1.

Évolution turbidimétrique de cultures de *Staph. pyogenes* souche Oxford. Chaque tube reçoit 9 ml de bouillon, contenant ou non de la streptomycine et 1 ml de culture non diluée de *Staph. pyogenes* âgée de 24 heures.

Tubes n ^{os}	Strepto- mycine, µg/ml	Temps d'incubation, en minutes											
		0	15	30	60	90	110	130	150	180	210	300	600
1	0	100	99	93	70	41	30	16	8	×			
2	0	100	96	90	65	39	27	11	5	×			
3	0	100	96	93	70	41	32	15	10	1	×		
4	100	100	101	99	75	68	62	55	50	45	40	27	×
5	100	100	98	97	73	62	58	50	45	42	35	21	×

Les chiffres sont obtenus par lecture directe sur le tambour de l'appareil; le signe × désigne un trouble trop intense pour que l'appareil le mesure sans être réglé d'une manière différente.

TABLEAU 2.

Évolution turbidimétrique de cultures d'*E. coli* K 12. Chaque tube reçoit 9 ml de bouillon, contenant de la streptomycine ou non, et 1 ml de culture d'*E. coli* K 12 âgée de 24 heures et diluée 1 : 10 en bouillon neuf.

Tubes n ^{os}	Strepto- mycine, µg/ml	Temps d'incubation, en minutes											
		0	15	30	60	90	110	130	150	180	210	300	600
1	0	100	96	92	84	68	56	38	25	16	5	×	
2	0	100	94	89	81	67	55	36	23	13	2	×	
3	0	100	97	91	85	69	55	36	28	16	5	×	
4	10	100	100	100	97	93	87	81	74	64	53	31	×
5	10	100	99	95	92	89	85	78	70	62	48	18	×

Même légende que pour le tableau 1.

les évolutions les plus dissemblables: on voit combien les divergences sont pourtant faibles. Des sub-cultures de ces tubes ont été préparées

TABLEAU 3.

Évolution turbidimétrique de cultures de *Myc. tuberculosis* souche B.C.G. Chaque tube reçoit 9 ml de milieu liquide de Dubos contenant ou non de la streptomycine et 1 ml de culture de *Myc. tuberculosis* âgée de 7 jours.

Tubes n ^{os}	Strepto- mycine, μg/ml	Temps d'incubation, en jours							
		0	1	2	3	4	5	7	10
1	0	100	81	63	48	35	26	×	
2	0	100	76	60	43	30	21	×	
3	0	100	79	61	47	31	21	×	
4	0.01	100	89	75	68	55	45	29	5
5	0.01	100	86	70	61	50	39	22	×

Même légende que pour le tableau 1.

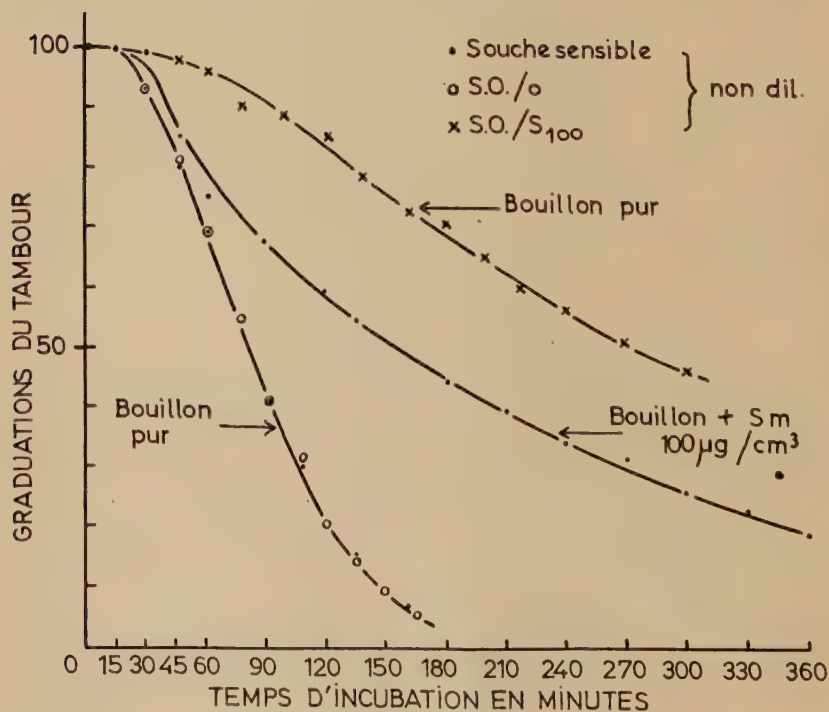


Fig. 1. Évolution turbidimétrique de cultures de *Staph. pyogenes* souche Oxford.

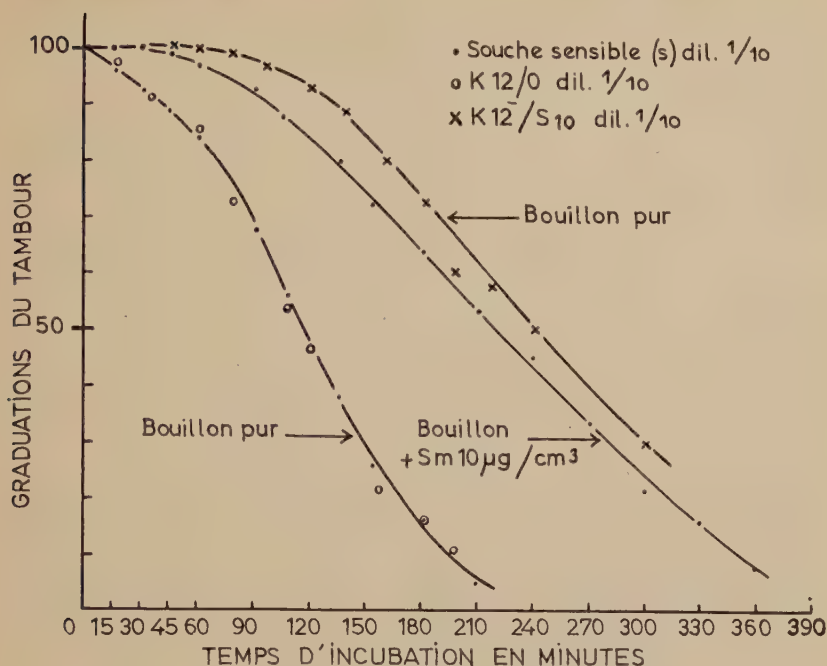


Fig. 2. Évolution turbidimétrique de cultures d'*E. coli* souche K 12.

et incubées dans les mêmes conditions que la culture-mère: le tube no. 3 est une de ces sub-cultures, et son évolution est étonnamment superposable à celle des cultures-mères.

Les figures 1 et 2 représentent certains de ces résultats sous forme graphique. Pour la commodité, nous appellerons les courbes obtenues : courbes de croissance (9).

Puisque l'intensité du trouble évolue dans le même sens que la richesse bactérienne (4), nos chiffres illustrent un phénomène connu: le développement des cultures bactériennes est constant lorsque les conditions restent constantes. La méthode turbidimétrique constitue seulement une méthode aisée et rapide pour suivre le rythme de la multiplication.

Le développement est altéré si l'on modifie les conditions de culture: par exemple, si l'on utilise comme semence des cultures d'âges différents, si la température d'incubation ou la composition du milieu de culture sont altérées, etc... les tubes n^{os} 4 et 5 des tableaux 1, 2 et 3 illustrent certaines modifications dues à l'introduction, dans le milieu, de concentrations non inhibitrices de streptomycine.

Ces tubes ont été choisis, parmi 10 tubes préparés de façon identique, parce que, de tous, c'étaient ceux dont l'évolution fut le plus dissemblable. On voit que cette évolution est, cependant, extraordinairement semblable.

Lorsqu'on modifie le nombre de bactériesensemencées dans un milieu sans streptomycine, la courbe de croissance subit un changement caractéristique: plus l'inoculum a été dilué, plus longtemps il faut attendre pour que le milieu se trouble de façon perceptible; mais la courbe ultérieure – c'est-à-dire sa partie mesurable – est strictement superposable à celle de la culture ensemencée abondamment. Ainsi, dans l'exemple du tableau 4, lorsqu'on ensemence au moyen d'une culture d' *E. coli* diluée 1:10, le trouble augmente déjà après 20 minutes d'incubation; lorsqu'on ensemence au moyen de la même culture diluée 1:100, la première augmentation du trouble n'est notée qu'après 80 minutes (soit un retard de 60 minutes); lorsqu'on ensemence au moyen de la culture diluée 1:1000, cette première augmentation du trouble n'est notée qu'après 140 minutes (soit un retard de 120 minutes). Les troubles s'accroissent davantage dans la suite, et on constate que les trois tubes passent par des valeurs identiques; seulement ces valeurs sont rencontrées par chacun à des moments différents, le retard persistant inchangé: l'évolution du tube ensemencé au moyen d'une culture diluée 1:100 reproduit l'évolution du tube ensemencé au moyen de la culture diluée 1:10, mais avec environ 60 minutes de retard; l'évolution du tube ensemencé au moyen de la culture diluée 1:1000 la reproduit à son tour, mais avec environ 120 minutes de retard.

TABLEAU 4.

Évolution turbidimétrique de cultures d'*E. coli* K 12.

Chaque tube reçoit 9 ml de bouillon pur et 1 ml de culture de 24 heures diluée en bouillon neuf.

Dilut. de la culture	Temps d'incubation, en minutes														
	0	20	40	60	80	100	120	140	160	180	200	220	240	260	280
1/10	100	96	92	85	75	60	47	31	23	18	8	×			
1/100	100	99	99	100	97	92	86	74	65	49	28	19	14	5	×
1/1000	100	100	101	99	100	100	100	96	92	88	76	66	52	30	21

Ce tableau ne représente que partiellement l'évolution du tube ensemencé au moyen de la culture diluée 1 : 1000; cette évolution continue régulièrement et aboutit en 320 minutes au trouble désigné par ×.

Un essai portant sur *Staph. pyogenes* souche Oxford, fournit des résultats semblables. Si on compare des tubes de 9 ml de bouillon ensemencés au moyen d'un ml de culture non diluée, diluée 1:10 ou diluée 1:100, on constate que l'évolution des courbes de croissance est identique, mais que la deuxième courbe reproduit les valeurs de la première avec 100 minutes de retard, et que la troisième les reproduit avec 280 minutes de retard.

Dans l'action de la streptomycine sur les souches sensibles, nous devons distinguer trois cas: 1) la streptomycine est utilisée à concentration non bactériostatique; 2) elle est utilisée à concentration bactériostatique; 3) des résistants apparaissent. Pour la facilité de notre étude, renversons cet ordre, et examinons d'abord le dernier cas.

A. Naissance de bactéries résistantes. En observant un grand nombre de cultures, nous avons pu assister par chance à la naissance même de races résistantes. Cela est arrivé moins rarement avec *Staph. pyogenes* qu'avec *E. coli*, et jamais avec *Myc. tuberculosis*. Dans chaque cas, les concentrations de streptomycine avaient été bactériostatiques. Après une longue période de latence, le milieu se troublait, mais la courbe turbidimétrique prenait, de suite, une allure différente de celle de la souche-mère sensible. La culture s'avérait résistante: ses subcultures toléraient sans peine la concentration de streptomycine bactériostatique pour la souche-mère. Un exemple est décrit dans le figure 3: deux tubes de 9 ml de

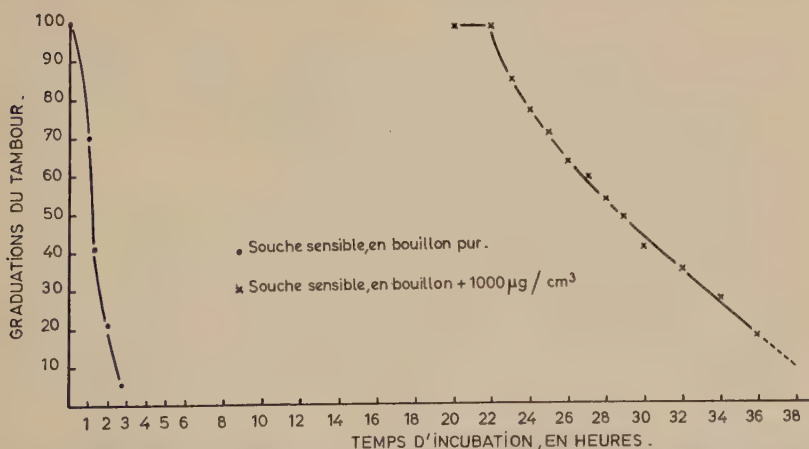


Fig. 3. Évolution turbidimétrique de *Staph. pyogenes* souche Oxford. Apparition d'une race résistante à la streptomycine.

bouillon, l'un pur, l'autre contenant 10000 μg de streptomycine, sont ensemencés au moyen d'un ml d'une culture de 24 heures de *Staph. pyogenes*, non diluée. Le tube témoin suit l'évolution habituelle. Le tube contenant la streptomycine (1000 $\mu\text{g}/\text{ml}$) reste pratiquement inchangé pendant 22 heures, puis se trouble; le développement y suit une courbe très différente.

La courbe nouvelle est héréditaire. En effet, si on ensemence la race résistante dans du bouillon sans streptomycine, cette courbe persiste inchangée; mais, naturellement, le temps de latence est considérablement écourté.

Toutes les races résistantes issues d'une même souche-mère sensible possèdent une courbe propre, différente l'une de l'autre. Il est impossible de dégager, de leur ensemble, une forme générale caractéristique de la résistance.

Si, dans le bouillon où l'on ensemence des germes modérément résistants, on a introduit de la streptomycine à concentration bactériostatique pour ces germes, ceux-ci réagissent comme les germes sensibles: ils subissent 1 ou 2 divisions, puis une lyse (4,7). A concentration non inhibitrice, l'antibiotique agit également comme sur les souches sensibles, dans les mêmes conditions: il modifie la courbe de multiplication, de façon héréditaire (voyez plus loin). Enfin, à toute concentration il peut faire naître des germes encore plus résistants.

B. Effet de la streptomycine à concentration bactériostatique. Nous pouvons être brefs et nous contenter de rappeler: 1° que les races résistantes apparaissent, le plus fréquemment lorsque la concentration est bactériostatique; 2° que, lorsqu'aucune résistance ne survient, les germes subissent un petit nombre de divisions, suivies de lyse (4,7).

C. Effets de la streptomycine à concentration bactériostatique. Même lorsque la concentration n'est pas bactériostatique, des germes résistants peuvent naître (5,6); mais il faut habituellement une série de passages dans des milieux additionnés de ces concentrations (10,3). Analysons une culture de premier passage au cours duquel aucune résistance n'apparaît.

Les tableaux 1, 2 et 3 et les figures 1 et 2 montrent que la streptomycine, à concentration non inhibitrice, modifie profondément les courbes de croissance. Le temps de latence, avant que le trouble

ne s'intensifie, est peu ou guère allongé. Au fur et à mesure que l'incubation se prolonge, les courbes de croissance s'écartent davantage de celles en bouillon pur (témoins); la vitesse de multiplication est généralement ralentie, et le ralentissement est identique dans les différentes cultures préparées et incubées de la même façon (ensemencements et concentrations de streptomycine identiques, etc . . .); les courbes d'une même souche sont alors superposables.

Le trouble maximum auquel parviennent les cultures développées en présence de concentrations non bactériostatiques de streptomycine, est presque toujours moins intense que celui des cultures témoins non additionnées de streptomycine. Les chiffres de nos tableaux et graphiques ne le révèlent pas: ils ne le pourraient pas puisqu'ils mesurent les modifications de chaque culture relativement à son propre point de départ (posé égal à 100); ils ne permettent pas du tout la comparaison des richesses microbiennes d'un tube à l'autre.

Nous avons mesuré celles-ci en comptant le nombre de cellules viables (dénombrement des colonies développées en gélose nutritive; méthode de la double couche de gélose de GRATIA (1,2)). En ensemantçant $3,6 \times 10^7$ cellules de *Staph. pyogenes* sensible par ml de bouillon, on trouve après 24 heures d'incubation à 37° C., 38×10^7 cellules viables par ml de bouillon pur (témoin), 31×10^7 cellules viables par ml de bouillon additionné de 10 μg de streptomycine et 14×10^7 cellules viables par ml de bouillon additionné de 100 μg de streptomycine. Les écarts ne sont pas considérables, mais se répètent dans tous les essais. On sait que les souches résistantes, en général, atteignent la même richesse microbienne en bouillon contenant de la streptomycine qu'en bouillon pur (8).

Revenons au ralentissement de la vitesse de multiplication des germes sensibles en milieu additionné de concentrations non bactériostatiques de streptomycine. Ce ralentissement n'est pas passager, les courbes le montrent; il persiste pendant toute l'incubation. La streptomycine gêne les premières divisions. Aucune des cellules filles ne parvient-elle à surmonter cette gêne? ou bien la streptomycine a-t-elle introduit un nouveau rythme de multiplication dans les cellules?

Reprenons les cultures du tableau 1 après 24 heures d'incubation à 37° C. Nommons SO/0 la race de *Staph. pyogenes* développée dans le tube n° 1, témoin (bouillon pur), et SO/Sm 100 la race du tube n° 4 (bouillon additionné de 100 μg de streptomycine par ml). Repre-

nous aussi les cultures du tableau 2, et nommons, de même, K 12/0 la race d' *E. coli* K12 développée dans le tube n° 1, témoin (bouillon pur) et K 12/Sm 10 la race du tube n° 4 (bouillon additionné de 10 μg de streptomycine par ml). Introduisons dans quatre tubes contenant 9 ml de bouillon pur, respectivement: 1 ml de SO/0 non dilué; 1 ml de SO/Sm 100 non dilué; 1 ml de K 12/0 dilué 1:10 en bouillon neuf; 1 ml de K 12/Sm 10 dilué 1:10. Portons les quatre tubes au bain-marie à 37° C. et mesurons leur trouble périodiquement. Les figures 1, 2 et 4 illustrent leur évolution.

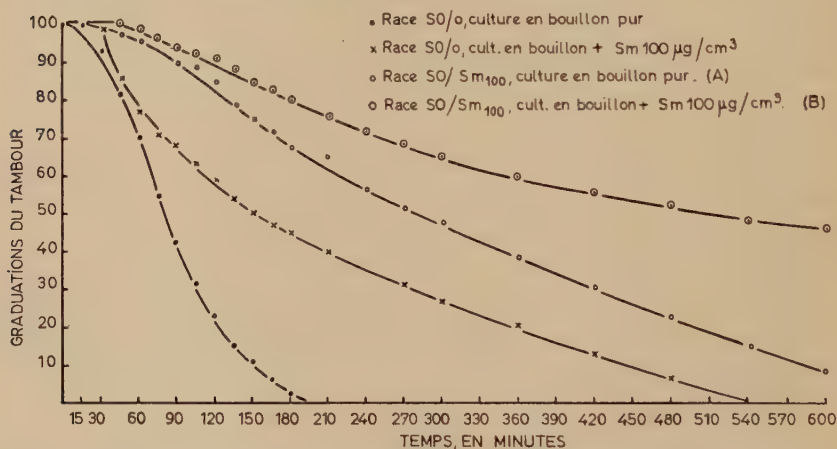


Fig. 4. Évolution turbidimétrique de *Staph. pyogenes* souche Oxford et de la race dérivée, SO/Sm 100 en bouillon pur ou additionné de streptomycine.

SO/0 se développe en retraçant, point par point, la courbe de *Staph. pyogenes* sensible, et se confond avec sa culture-mère. Par contre, la courbe de SO/Sm 100, bien que cette race se développe en bouillon sans streptomycine, ne ressemble en rien à la courbe de SO/0. En fait, elle reproduit exactement la courbe de croissance de la culture dont elle est issue, c'est-à-dire celle de *Staph. pyogenes* sensible se développant en bouillon additionné de 100 μg de streptomycine par ml. Elle en diffère cependant par une chose: sauf à leur origine, les courbes ne se confondent pas, mais sont équidistantes, celle de SO/Sm 100 ayant un retard constant sur l'autre (retard de 80 minutes environ).

Les mêmes observations se répètent pour K 12/0 et K 12/Sm 10. La courbe de croissance de K 12/0 se confond avec celle de la souche-mère *E. coli* ensemencée en bouillon pur. Celle de K 12/Sm 10,

quoique la multiplication ait lieu en bouillon pur, reproduit celle de *E. coli* dans le bouillon additionné de 10 μ g de streptomycine par ml; elle n'en diffère que par un retard de 15 minutes environ.

En somme, les races SO/Sm 100 et K 12/Sm 10 ont conservé, en bouillon pur, le rythme de développement imposé par la streptomycine dans les cultures dont elles sont issues.

Mais SO/Sm 100 et K 12/Sm 10 sont, on l'a vu, moins riches en cellules viables que les cultures témoins SO/0 et K 12/0. Aussi avons-nous tenté d'égaliser les troubles avant d'ensemencer les tubes de bouillon pur. Dans ce but, les cultures SO/Sm 100 et K 12/Sm 10 sont soumises à une centrifugation énergique (10 minutes à 6000 tours par minute); les sédiments microbiens sont remis en suspension dans une quantité telle de surnageant que les troubles soient équivalents aux témoins, SO/0 et K 12/0. Ces suspensions sont ensemencées, comme précédemment, dans du bouillon pur: comme il fallait s'y attendre, les temps de latence, avant qu'augmente le trouble, sont raccourcis, mais les courbes elles-mêmes restent inchangées, c'est-à-dire, retracent les courbes des souches-mères sensibles dans du bouillon contenant 100 ou 10 μ g de streptomycine par ml, respectivement. Le retard de SO/Sm 100 par rapport à *Staph. pyogenes* sensible en bouillon additionné de 100 μ g de streptomycine par ml, passe ainsi de 80 minutes à 15 minutes; celui de K 12/Sm 10 en bouillon pur par rapport à *E. coli* en bouillon additionné de 10 μ g par ml, passe de 15 à 5 minutes.

Ce qui subsiste du retard peut-il s'expliquer par la présence de streptomycine dans l'inoculum? Celui-ci contient, en effet, la streptomycine introduite dans la culture précédente. Pour nous en débarrasser, centrifugeons la culture SO/Sm 100 et remettons le sédiment en suspension dans du bouillon pur, neuf, en volume tel que le trouble soit équivalent à celui de la culture SO/0. Ensemençons 1 ml de cette suspension dans 9 ml de bouillon pur. La courbe de croissance reste identique à celle de *Staph. pyogenes*, souche sensible, dans du bouillon additionné de 100 μ g de streptomycine par ml, mais elle n'aura plus qu'un retard de 5 minutes environ sur celle-ci. Il est donc possible — mais non certain — que le retard soit dû uniquement à la streptomycine de l'inoculum.

Les races nouvelles, SO/Sm 100 et K 12/Sm 10, sont restées aussi sensibles à l'action bactériostatique de la streptomycine que les souches-mères. En effet, les concentrations bactériostatiques sont les suivantes:

Staph. pyogenes

Souche-mère:	10 ⁷ cellules par ml:	125 µg/ml
	10 ³ " "	9 "
SO/Sm 100 :	10 ⁷ " "	125 "
	10 ³ " "	10 "

E. coli

Souche-mère:	10 ⁷ cellules par ml:	31 µg/ml
	10 ³ " "	6 "
K 12/Sm 10 :	10 ⁷ " "	31 "
	10 ³ " "	6 "

Les races SO/Sm 100 et K 12/Sm 10 ont néanmoins acquis des caractères nouveaux qui se manifestent dans leurs courbes de croissance. Ces caractères sont héréditaires: après trois subcultures en bouillon pur, chaque race avait conservé sa courbe propre (ce qui montre, d'ailleurs, que le tracé de ces courbes — non pas le retard — n'est pas dû à la streptomycine entraînée avec la semence).

A leur tour, les races SO/Sm 100 et K 12/Sm 10 peuvent être cultivées en présence de streptomycine (tab. 5). Ensemençons, par exemple, 1 ml de SO/Sm 100 non dilué (tube n° 4 du tableau 1, après 24 heures à 37° C.) dans des tubes contenant 9 ml de bouillon sans streptomycine (tube témoin, A) ou additionnés de 1000 µg de streptomycine (tube B, où la concentration de l'antibiotique sera de 100 µg/ml). La courbe de croissance dans le tube A nous est connue maintenant; quant à celle du tube B, elle s'écarte à nouveau de celle du tube A: la vitesse de multiplication est encore réduite (fig. 4). La culture du tube B, elle aussi, est restée sensible à l'action bactériostatique de la streptomycine, autant que la souche originelle de *Staph. pyogenes*. Mais elle a acquis des propriétés nouvelles: sa courbe de croissance le démontre. En effet, si on ensemence 1 ml du tube B dans un nouveau tube de bouillon pur, sans streptomycine, le développement se fait, non plus suivant la courbe du tube A, mais bien suivant celle du tube B: une nouvelle race est née dans le tube B, elle possède une courbe de croissance propre et héréditaire.

L'étude de K 12/Sm 10 montre des faits semblables: l'introduction de 10 µg de streptomycine dans le bouillon où l'on ensemence, modifie sa courbe de croissance, la vitesse de multiplication est réduite. Cette modification est définitive, elle persiste en l'absence de streptomycine, et est héréditaire.

Si l'on compare l'influence des concentrations non bactériostatiques de la streptomycine sur les bactéries sensibles et sur des

bactéries résistantes, on est frappé par une différence fondamentale: avec les bactéries sensibles, l'antibiotique allonge peu le temps de latence qui précède l'augmentation du trouble, mais il modifie (ralentit) la vitesse de multiplication; avec les bactéries très résistantes, au contraire, il allonge considérablement le temps de latence, mais ne modifie guère la vitesse de multiplication (8). Il se peut que ces différences soient attribuables aux concentrations de streptomycine utilisées: les concentrations non bactériostatiques pour les germes sensibles sont évidemment beaucoup plus petites que celles que tolèrent des races très résistantes.

Des passages répétés d'une souche sensible dans du bouillon additionné de streptomycine à concentration constante, non bactériostatique, peuvent, on le sait, faire apparaître la résistance (10, 3). On peut se demander si ces passages ne s'accompagneraient pas d'une altération progressive des courbes de croissance: après un certain nombre de passages, ne verrait-on pas, à chaque nouveau passage, la streptomycine agir davantage sur le temps de latence et moins sur la vitesse de multiplication?

TABLEAU 5.

Évolution turbidimétrique de la race obtenue par l'action de la streptomycine sur *Staph. pyogenes*.

Souches et races	Strepto- mycine μg/ml	Temps d'incubation, en minutes																	
		0	15	30	45	60	75	90	105	120	135	150	165	180	200	270	300		
SO/0	0	100	100	93	82	70	55	41	32	21	15	10	6	×					
	100	100	100	99	84	76	71	68	63	59	54	50	47	45	40	31	27		
SO/S100	0	100	100	99	98	96	91	90	89	86	79	75	73	71	67	51	47		
	100	100	100	100	99	97	96	93	92	91	88	84	80	78	70	62	59		

SO/0 et SO/S100 sont tous deux ensemencés à raison de 1 ml de culture non diluée par tube; en réalité, la première culture est 4 à 5 fois plus riche que la dernière.

CONCLUSIONS.

- 1) La streptomycine, en concentration sub-bactériostatique, modifie la courbe de croissance des bactéries sensibles en milieu liquide. Elle ralentit la vitesse de multiplication. Elle a peu d'action sur la période de latence qui précède l'augmentation du trouble.
- 2) Ces modifications sont héréditaires et persistent dans les subcultures en milieu liquide sans streptomycine. Elles ne s'accompagnent cependant d'aucune résistance à l'action bactériostatique de l'antibiotique.

- 3) L'addition de streptomycine aux subcultures provoque à nouveau des effets analogues et modifie encore la courbe de croissance. Ces nouvelles modifications sont également héréditaires.
- 4) Toutes ces modifications semblent différentes de celles qu'on observe lorsque des races résistantes se développent en présence de concentrations non bactériostatiques de streptomycine.

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(Istituto di Industrie Agrarie, University of Perugia, Italy).

A SIMPLE CHROMATOGRAPHIC TECHNIQUE FOR THE IDENTIFICATION OF ETHYL ACETATE AND OTHER ESTERS IN A CULTURAL MEDIUM

by

CORRADO CANTARELLI

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The formation of so called odorous esters is a peculiar, interesting characteristic of the fermentation caused by various micro-organisms and may have a diagnostic value specially in the identification of some yeasts (LODDER and KREGER-VAN RIJ, 1952). For this purpose, their determination in a cultural medium is usually conventionally trusted to smell alone. Ethyl acetate is the ester most abundantly found (PEEL, 1951) and its odor is generally easy to recognize. However, an exact determination and identification of these compounds by distillation and hydrolysis is not always suitable and easily accomplished.

It has been found to be convenient in the study of various species of fungi and bacteria to employ a simple ester identification technique by examining their corresponding hydroxamic acids. In fact, the formation of these derivatives is a specific reaction of the esters and therefore, their separation can be chromatographically correctly ascertained, revealing them to be ferric complexes, as found under other conditions by THOMPSON (1950) and by TABACHNICK (1953).

The following procedure was adopted: 1—2 ml of the culture medium were mixed with 20 ml of ethyl ether followed by 2 ml of alkaline hydroxylamine solution prepared immediately before use by equal volumes of 5% $\text{HCl-NH}_2\text{OH}$ and of 3 N NaOH both in methanol and the solution filtered to remove the NaCl. After an hour, 0.15 ml glacial acetic acid was added and the mixture shaken. The ether was then evaporated at 35—40° C. (preferably *in vacuo*). Aliquots of 20—40 microliters were used for the chromatographic examination, using for the ascending or the descending technique

Whatman # 1 paper and as solvent, amyl alcohol-acetic acid-water (74 : 19 : 50). After the development, the paper was dried at room temperature and sprayed with a saturated solution of FeCl_3 in *n*-butanol. The spots corresponding to the ferric hydroxamate complex appear clearly colored in violet so that even 0.5 microgram of the esters is identifiable.

Some results of yeasts cultures in malt extract of 3 days are illustrated in figure 1.

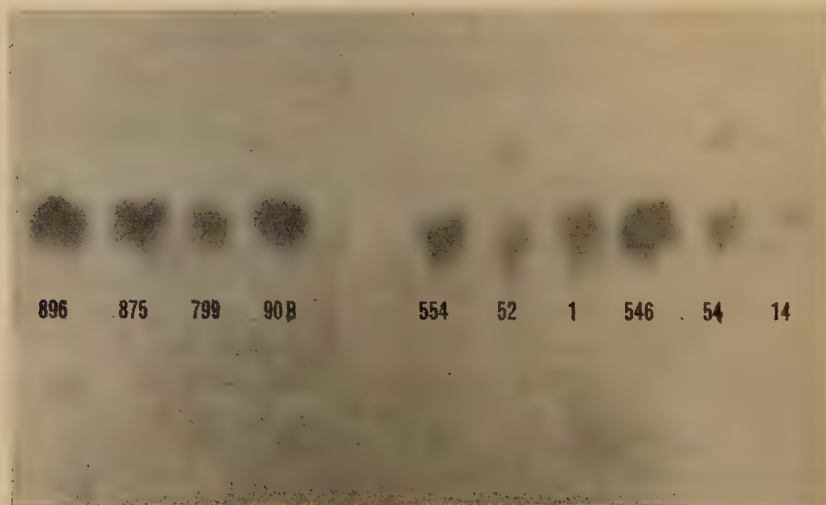


Fig. 1. Chromatographic evidence of ester production by various yeast cultures. From left to right: 896 *Brettanomyces* sp.; 875 *Candida pulcherrima*; 799 *Debaryomyces tyrocola*; 90B *Kloeckera apiculata*; 554 *Torulaspora rosei*; 52 *Hansenula anomala*; 1 *Saccharomycodes Ludwigii*; 546 *Saccharomyces uvarum*; 54 *Hansenula fermentans*; 14 *Saccharomyces ellipsoideus*.

The chromatogram shows that all the yeasts examined produce ethyl acetate and in addition, the genus *Hansenula* produces butyl esters as well.

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(Laboratoire annexé au Service de Dermato-Syphiligraphie des Hôpitaux
Universitaires de Bruxelles).

PRÉSENCE DE GRANULATIONS DU TYPE POLAIRE DANS LES CELLULES BACTÉRIENNES D'*E. COLI* ET DU *B. SUBTILIS* SOUS L'INFLUENCE DES DÉTERSIFS

par

A. DELMOTTE

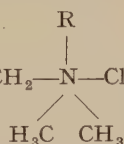
(Reçu le 27 Avril 1955).

Les détersifs, encore appelés "epiergiques", "tensio-actifs" ou "surfactants" sont des substances qui, très généralement à des concentrations de 0.5-1 g par litre en solution aqueuse, ont la propriété d'abaisser la tension superficielle. Ils se composent tous d'une longue chaîne hydrophobe terminée par un chaînon hydrophile court. Suivant la charge électrique de la longue chaîne, les détersifs peuvent être classés en *anioniques* comme le lauryl-sulfate de soude, et en *cationiques* comme le chlorure de cétyl-pyridinium. Il existe aussi un groupe important de détersifs *non-ioniques*.

L'action de certains détersifs, tant anioniques et cationiques, que non-ioniques sur une suspension en eau physiologique d'*Escherichia coli* et de *Bacillus subtilis* se traduit par la mise en évidence de granulations dans les cellules bactériennes. Ces granulations sont visibles par le simple examen entre lame et lamelle au microscope ordinaire. Le dispositif de contraste de phase ou l'addition aux solutions de détersifs de colorants basiques tels que Bleu de Nil, fuchsine basique, brun Bismarck ou vert Janus (voir fig. 1 et tableau 1) permet en général de voir mieux encore ces granulations.

DÉTERSIFS UTILISÉS:

I. **Aminol.** Composé cationique de formule $C_6H_5CH_2-N-Cl.H_2O$



où R représente un groupe alkyle complexe, de pureté 96% pour le *p* aduit „pur” et 85% pour le produit „technique”. Union Chimique Belge.

2. **Chlorure de cétyl-pyridinium.** Composé cationique. Tensia, Liège.
3. **Chlorure de cétyl-diméthyl-benzyl-ammonium.** Composé cationique. Tensia, Liège.
4. **Belsam III/50,** hexastéarate de poly-glycol. Composé non-ionique. Union Chimique Belge.
5. **Emullat S 25,** alkylpolyéthylène-glycol de formule $R-O (C_2H_4O)_nH$. Composé non-ionique. Union Chimique Belge.
6. **Emullat CO₁₀,** éther de polyéthylène-glycol, de formule $R-O (C_2H_4O)_nH$. Composé non-ionique. Union Chimique Belge.
7. **Emullat 29,** alkyl-aryl-polyéthylène-glycol de formule $R-O (C_2H_4O)_nH$. Composé non-ionique. Union Chimique Belge.
8. **Emullat R 40,** éther polyéthylène-glycol d'un glycéride d'acide gras de formule $R[(C_2H_4O)_nH]_3$. Composé non-ionique. Union Chimique Belge.
9. **Tensomel P.E. 11,** condensat de mono-éthanolamine sur acide gras. Composé non-ionique. Tensia, Liège.
10. **Tensolase D 30,** condensat d'oxyde d'éthylène sur alcool oléique. Composé non-ionique. Tensia, Liège.
11. **Tensolase 45.** Tensia, Liège.
12. **Tensolase D 60,** condensat d'oxyde d'éthylène sur alcool $C_{16}-C_{18}$. Composé non-ionique. Tensia, Liège.
13. **Tensophène D 42,** condensat d'oxyde d'éthylène sur nonylphénol. Composé non-ionique. Tensia, Liège.
14. **Tensophène H 10.** idem. Tensia, Liège.
15. **Emullat 06,** éther polyéthylène-glycol d'acide gras de formule $R-COO (C_2H_4O)_nH$. Composé non-ionique. Union Chimique Belge.
16. **Base LP12,** éther de polyéthylène-glycol. Composé non-ionique. Union Chimique Belge.
17. **Ucépal, DC,** éther-alkyl-aryl-polyéthylène-glycol de formule générale $R-O(C_2H_4O)_nH$. Composé non-ionique. Union Chimique Belge.
18. **Tensaryl 80 B,** Sulfonate sodique de dodécylbenzène. Composé anionique. Tensia, Liège.
19. **Tensopol U.S.P.,** Sulfate sodique d'alcool $C_{12}-C_{14}$. Composé anionique. Tensia, Liège.
20. **Tensiagex DC 12,** Sulfate sodique d'éther polyglycolique. Composé anionique. Tensia, Liège.
21. **Tensadine D 119,** Sulfate sodique d'alcool $C_{16}-C_{18}$. Composé anionique. Tensia, Liège.
22. **Tensopol MG,** Sulfate magnésique d'alcool $C_{12}-C_{14}$. Composé anionique. Tensia, Liège.
23. **Tensatil,** Sulfate sodique d'alcool C_8-C_{10} . Composé anionique. Tensia, Liège.
24. **Synkapon.** Composé non-ionique. Union Chimique Belge.

TECHNIQUE

L'E. coli est une souche isolée d'une lésion cutanée; *B. subtilis* est la souche B₂ de l'Institut Pasteur de Paris et nous a été obligeamment fournie par le Docteur W. MUTSAARS du Service de Bactério-

logie de la Faculté de Médecine de Bruxelles, que nous remercions ici.

Une culture de 24 heures d'*E. coli* ou et *B. subtilis* sur gélose inclinée a été utilisée dans tous les cas. Après 24 heures, *B. subtilis* ne fournit pas de spores. Le contenu d'une ôse de platine de cette culture est dispersée sur une lame porte-objet dans deux à trois gouttes d'une solution de détersif à 1 g 0/00 additionnée ou non d'un colorant basique: bleu de Nil, vert Janus, brun Bismarck, ou fuchsine basique. L'examen se fait extemporanément entre lame et lamelle.

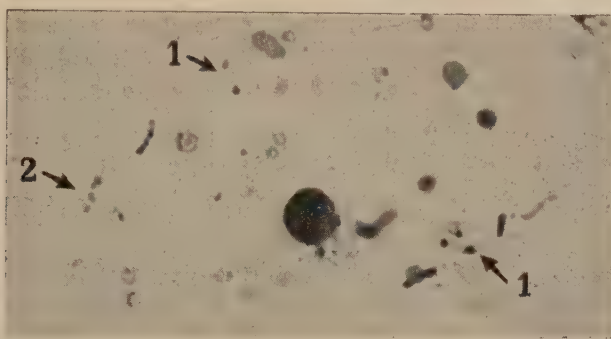


Fig. 1. Granulations polaires d'*E. coli*. 1. Granulations bipolaires. 2. Formation centrale tortueuse.

MORPHOLOGIE DES GRANULATIONS.

Les granulations mises en évidence par les détersifs dans les colibacilles se présentent généralement aux deux pôles de la cellule; lorsqu'il s'agit d'un bacille un peu allongé, il existe aussi une granulation centrale, et quand apparaît une forme en fuseau, plusieurs granulations se voient à intervalles réguliers, ce qui est l'aspect habituellement observé dans les cellules du *B. subtilis* lorsqu'elles sont soumises à l'action des tensioactifs. Toutefois, chez le colibacille, et plus rarement, on peut voir quelques formations centrales, souvent tortueuses. Ces images sont comparables à celles observées par BONDARENKO-ZOZULINA (6): les granulations polaires sont celles des cellules jeunes, les formations centrales représentent les modifications des granulations polaires sous diverses circonstances d'altération et notamment l'âge.

Les granulations polaires observées dans les cellules du colibacille par BONDARENKO-ZOZULINA (6) ont été retrouvées en 1950 par

WACHSTEIN et PISANO (29) dans les cellules d' *E. coli*, de *Shigella dysenteriae*, de *Salmonella typhosa* et *Gaffkya tetragena* à l'aide de

TABLEAU 1.

Action des détersifs et des colorants vitaux sur les cellules d' *E.coli*.

Détersifs (1 g 0/00)	Brun Bismarck	Fuchsine	Bleu Nil	Vert Janus
Aminol, technique	1	5	4	1
Aminol, pur	2	1	3	5
Chlorure de cétyl- pyridinium	2	6	5	5
Chlorure de cétyl-di- méthyl-benzyl- ammonium	4	3	1	4
Belsam III/50	2	1	3	3
Emullat S 25	3	4	3	3
Emullat CO ₁₀	3	3	4	6
Emullat 29	1	5	3	1
Emullat R 40	3	4	5	3
Tensomel P.E. 11	3	4	4	4
Tensolase D 30	1	3	3	5
Tensolase 45	2	4	4	3
Tensolase D 60	6	5	3	6
Tensophène D 42	6	3	4	5
Tensophène H 10	5	3	3	4
Emullat 06	5	1	4	1
Base LP 12	1	1	3	3
Ucépal DC	5	1	3	5
Tensaryl 80 B	5	5	5	1
Tensopol U.S.P.	5	5	5	2
Tensiagex DC 12	1	5	5	1
Tensadine D 119	5	5	3	1
Tensopol MG	1	5	1	3
Tensatil	5	4	3	1
Synkapon	5	5	6	5
Témoin: Eau distillée	6	4	3	6

Légende: 1: seules les granulations sont visibles.

2: coloration douteuse ou faible du corps bactérien, et granulations visibles.

3: coloration du corps bactérien et coloration rouge des granulations quelque soit le détersif employé.

4: coloration du corps bactérien, sans différenciation.

5: absence de coloration.

6: coloration douteuse ou faible du corps bactérien; pas de granulations visibles.

la coloration histologique de Gomori pour la mise en évidence des phosphatases (FIRKET (11)).

ACTION DE LA CHALEUR SUR LES CELLULES BACTÉRIENNES.

Au cours d'expériences témoins, et en l'absence de détersifs, nous avons pu constater que le chauffage simple à 55°C. pendant une heure permet de mettre en évidence, mais d'une façon moins nette et moins constante, des granulations qui n'existaient pas dans les cellules non chauffées. On sait qu'un tel chauffage dissout en partie l'acide ribonucléique bactérien (CALIFANO (7)).

ACTION ENZYMATIQUE SUR LES GRANULATIONS.

a. Action de la désoxyribonucléase.

Ces granulations représentent-elles des "noyaux" ou des "nucléïdes" bactériens (1-5, 8-10, 12-28, 30)? En effet, la présence de granulations du type nucléaire avait déjà été décrite dans les cellules du bacille dysentérique de Flexner sous l'influence de deux détersifs anioniques: le lauryl-sulfate et le lauryl-sulfonate de soude (8, 9).

Si, suivant BOIVIN, nous soumettons les suspensions d' *E. coli* et de *B. subtilis* à l'action de la désoxyribonucléase à 55° C. pendant une heure et qu'ensuite ces suspensions sont soumises à l'action des détersifs, nous constatons que les granulations persistent, tandis que les zones centrales classiquement occupées par les "noyaux" ou "nucléïdes" apparaissent optiquement vides.

Ces granulations ne sont donc apparemment pas constituées de matériel nucléaire.

b. Action de la ribonucléase.

La ribonucléase, employée de la même manière que la désoxyribonucléase, c'est-à-dire à 55° C. pendant une heure, détermine la disparition de grandes parties des cellules bactériennes traitées, mais laisse intacte les granulations observées sous l'action des détersifs.

c. Action simultanée de la ribonucléase et de la désoxyribonucléase.

L'emploi simultané de la ribonucléase et de la désoxyribonucléase à 55°C. pendant une heure altère profondément la structure intime des cellules bactériennes, mais laisse inchangées les granulations révélées par l'action des détersifs. Il faut noter toutefois que

ces granulations sont plus visibles mais peut-être s'agit-il là d'un effet optique de contraste.

d. Action de l'hyaluronidase seule ou combinée avec la ribonucléase et la désoxyribonucléase.

L'hyaluronidase seule, l'hyaluronidase combinée à la ribonucléase ou à la désoxyribonucléase, l'hyaluronidase combinée à ces deux dernières enzymes ne modifie pas l'aspect des granulations bactériennes.

e. Action de la salive humaine diluée au quart ou de l'amylase (U.C.B.).

1. Action sur les granulations du *B. subtilis*.

Les granulations que les détersifs font apparaître dans les cellules du *B. subtilis* disparaissent lorsque les suspensions bactériennes sont traitées à 37°C. pendant une demi-heure soit par la salive humaine diluée au quart, soit par l'amylase au centième.

2. Action sur les granulations d'*E.coli*.

L'amylase ou la salive agissant sur les suspensions bactériennes pendant cinq heures, à 37°C. fait baisser le taux des bacilles présentant des granulations sous l'action des détersifs de 95% à 35% pour tous les détersifs envisagés, sauf pour un détersif cationique: l'aminol pur et un détersif non-ionique: l'émullat 06.

Dans le cas particulier de ces deux détersifs, il est intéressant de constater que les granulations disparaissent lorsque les suspensions bactériennes ont été préalablement traitées par la désoxyribonucléase et la ribonucléase à 55° C. pendant une heure, puis par la salive au quart ou l'amylase au centième à 37° C. pendant une demi-heure, la désoxyribonucléase et la ribonucléase faisant toujours partie du mélange enzymatique.

f. Le problème de la digestion enzymatique des granulations d'*E.coli*.

Pour réduire le nombre des bacilles présentant des granulations de 95% à 2% (numérations sur 500 bacilles), il faut une action de la désoxyribonucléase et de la ribonucléase pendant une heure à 55° C., suivie d'une action de l'hyaluronidase à 37°C. pendant une heure, les deux premiers enzymes étant toujours présentes, et enfin d'une digestion de 4 heures à 37° C. par l'amylase (U.C.B.) au centième ou la salive humaine diluée au quart ajoutée au mélange enzymatique.

Dans les expériences témoins, nous avons tenté de modifier les conditions d'expérience en essayant l'action de la désoxyribonucléase à 55° C. pendant une heure, puis à 37° C. pendant cinq heures, celle de la ribonucléase seule ou associée à la désoxyribonucléase, celle de ces deux enzymes avec l'hyaluronidase dans les mêmes conditions d'expérience, celle de la salive ou de l'amylase agissant d'une façon prolongée Toutes ces expériences, y compris l'action unique de la température se soldèrent par un échec, sauf les deux expériences que nous avons relatées plus haut, c'est-à-dire le cas des granulations révélées par l'action de l'aminol et de l'émullat 06. Notons encore que de multiples lavages de suspension d'*E. coli* ou de *B. subtilis* suivis ou non d'un traitement à la désoxyribonucléase ou à la ribonucléase à 55 °C. pendant une heure ne permettent pas de faire disparaître les granulations révélées par les détersifs.

g. Modifications de la coloration „vitale” du *B. subtilis* soumis à l'action des détersifs après digestion enzymatique.

Le *B. subtilis* soumis à l'action des mélanges Ucépál DC + fuchsine basique, Emullat 29 + brun Bismarck, Tensopol Mg + bleu de Nil, Tensaryl + vert Janus, ne montre que des granulations nettement visibles et de teinte rouge quelque soit le colorant employé. Le corps bactérien lui-même n'est pas coloré. Après action de l'amylase à 37°C. pendant une demi-heure, le bacille est coloré d'une manière homogène dans son entièreté, en rouge par la fuchsine, en bleu par le bleu de Nil, en vert-bleu par le vert Janus et en brun par le brun Bismarck.

Les systèmes Emullat 06 + vert Janus, Emullat 06 + fuchsine basique, Base L.P. 12 + fuchsine basique, Aminol technique + brun Bismarck, Tensopol Mg + brun Bismarck, Tensopol Mg + brun Bismarck, Aminol Technique + vert Janus, Tensolase D 30 + brun Bismarck ne mettent également en évidence que les granulations du *B. subtilis* sans colorer le bacille lui-même. Après action de l'amylase ou de la salive, les granulations ont disparu et les bacilles ne prennent pas le colorant.

Dans le système Base L.P. 12 + vert Janus, les granulations apparaissent en rouge, tandis que le bacille est légèrement coloré en vert-bleu. Après action de la salive ou de l'amylase, les bacilles ne sont plus colorés et les granulations ont disparu.

Dans le système Base L.P. 12 + brun Bismarck les granulations

apparaissent en rouge sur fond non coloré. Après action de la salive ou de l'amylase, le bacille se colore irrégulièrement en brun et les granulations ont disparu.

NATURE DES GRANULATIONS POLAIRES.

Les granulations observées sous l'action des détersifs dans *E. coli* et le *B. subtilis* paraissent de constitution chimique proche, mais non identique. En effet, tandis que celles du *B. subtilis* disparaissent facilement sous l'action de la salive au quart ou de l'amylase (U.C.B.) au centième, les granulations colibacillaires exigent pour disparaître l'action simultanée de quatre enzymes: la desoxyribonucléase, la ribonucléase, l'hyaluronidase et l'amylase.

On peut donc émettre l'hypothèse de la nature vraisemblablement glycogénique des granulations de *B. subtilis*, tandis que dans celles du colibacille, le substrat glycogénique doit être associé à d'autres substances de nature mucopolysaccharique, nucléoprotéique ou protéinique. Il est intéressant de rappeler que PETRONELLI (24) a pu mettre en évidence le noyau bactérien grâce à l'action de la salive humaine qui contient de la ribonucléase, du lysozyme et souvent de l'hyaluronidase.

La théorie émise en 1949 par BONDARENKO-ZOZULINA (6) suivant laquelle les noyaux de ROBINOW et PESHKOFF sont des artefacts postmortem représentés par les altérations des granulations polaires observées in vivo nous semble controuvée par les expériences nombreuses consacrées à la question, notamment les examens au contraste de phase de TULASNE (27) et nos propres observations.

Par contre, nous pouvons nous rattacher aux conclusions de WACHSTEIN et PISANO (29): les granulations polaires coexistent avec les noyaux bactériens et résistent à l'action de la désoxyribonucléase, de la ribonucléase, de la pepsine et de la trypsine.

R é s u m é.

L'action de détersifs des groupes anioniques, cationiques et non-ioniques à la concentration d'1 g 0/00, permet, dans certains cas, la mise en évidence de granulations dans les cellules bactériennes d'*E. coli* et du *B. subtilis*. Ces granulations se voient sans coloration, mais elles sont généralement plus visibles au contraste de phase ou par l'emploi de colorants basiques tels que fuchsine basique, bleu de Nil, vert Janus et brun Bismarck. Elles peuvent également être vues par l'action du bleu de Nil en l'absence de détersifs.

Ces granulations sont du type polaire et peuvent être assimilées aux granulations polaires de BONDARENKO-ZOZULINA (6).

Elles résistent à l'action de ribonucléase, de la désoxyribonucléase et de l'hyaluronidase. L'amylase seule fait disparaître les granulations observées dans les cellules du *B. subtilis*, tandis qu'il faut l'action simultanée et prolongée d'un mélange de désoxyribonucléase, ribonucléase, hyaluronidase et amylase pour faire disparaître la plupart des granulations d'*E. coli*.

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A NEW, CRYSTALLINE, ANTIBIOTIC SUBSTANCE PRODUCED BY *MOLLISIA* SPECIES (DISCOMYCETES)

by

J. GREMMEN

(Received April 13, 1955).

INTRODUCTION.

During systematic work in the Inoperculate Discomycetes a great many species were cultured in order to investigate their biology in vitro, since culture-work has proved to be very useful and sometimes necessary for taxonomics to demonstrate an existing relationship with a certain imperfect stage. These trials were, moreover, performed for studying the agencies which influence the development of apothecia under controlled conditions. Part of this work has already been published in a preliminary paper (GREMMEN, 1952).

At first members of several discomycete families were studied but it was soon decided to concentrate attention on the *Mollisioideae* sensu Nannfeldt, mainly species of the genus *Mollisia* (Fr.) Karst., since many of them inhabit wood and contribute to its decay.

Information on culture-work in the genus *Mollisia* is very scanty and there is hardly any case in which a conidial stage has been proved with certainty. Up till now the present author too has been unable to detect any in vitro.

Cultures were obtained in the manner described in an earlier paper (GREMMEN, 1952). Best results were got on maltagar, although cherry decoction agar may also be suitable for increasing mycelial growth, whereas on maltagar the chance of getting apothecia is much higher.

In the course of isolating different strains of *Mollisia*, two isolates, 69a and 71b, were acquired, producing a characteristic yellow substance on the surface of the agar. This phenomenon was observed a week after transferring small blocks of agar to plates with malt-

agar. Lemon- to sulphur-like masses of crystals, gradually increasing, were noticed.

About one year later a third strain (170) was isolated. In this strain development of clusters of yellow crystals was considerably higher than was ever observed in isolates 69a and 71b. Again seven to eight days after making transfers to other maltagar plates, the characteristic yellow substance was developed and observed in the periphery of the colony. All these observations stimulated further study on the nature of this substance.

For this purpose subcultures of strains 69a and 71b were handed over to Prof. Dr O. F. UFFELIE, Pharmaceutisch Laboratorium, Rijksuniversiteit, Utrecht. After two years UFFELIE informed us that the yellow substance appeared to be a new and hitherto undescribed chemical substance. He proposed to designate it „mollisine”.

Subcultures of strain 170 later were sent to the Institute for Organic Chemistry T.N.O. at Utrecht (Dr G. J. M. VAN DER KERK and Dr A. KAARS SYPESTEYN) for a chemical investigation of the compound. “Mollisine” has been obtained in larger amounts and its molecular formula established. Analysis and absorption spectrum confirmed the view of UFFELIE that we are dealing here with an unknown antibiotic compound.

The results of investigations on the antibiotic and the chemical properties of “mollisine” will be published separately by the Institutes mentioned above.

STUDIES ON THE ANTAGONISTIC PROPERTIES OF MOLLISINE.

It appears that this substance which crystallises so freely in pure cultures of the fungus — the author never saw them in nature — has pronounced antifungal properties. This antagonistic action has been demonstrated in the laboratory with a series of fungi, viz., *Fomes annosus* (Fr.) Cke., *Dothichiza populea* Sacc. & Bri., *Sclerotinia trifoliorum* Erikks., *Sclerotinia minor* Jagger, *Pollaccia radiosa* (Lib.) Bald. & Cif., *Cladosporium* spec. (614 and x) and *Penicillium* spec. (615).

A series of plates of maltagar were excentrically inoculated with small blocks of agar, every plate with one block from strain 170. Four to ten days afterwards an equal block of agar of the fungus to be tested was excentrically placed too in the same petri-dish. but to the opposite side. These tests were performed with the above mentioned fungi. Strain 170 was always given a start, because of

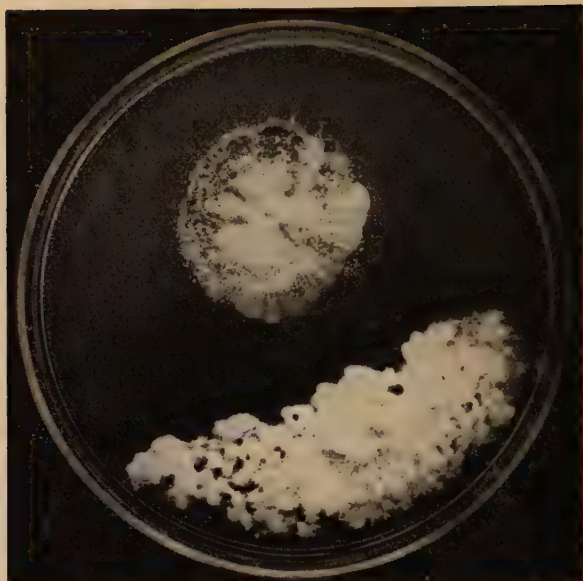


Fig. 1. Antagonistic action between strain 170 and *Dothichiza populea*. Black background. On maltagar (Foto J. B. W. WEG).



Fig. 2. Antagonistic action between strain 170 and *Fomes annosus*. Black background. On maltagar (Foto J. B. W. WEG).

the quicker growth of the other organisms. After a fortnight a strong inhibiting effect was noticed. The periphery of strain 170 was covered with numerous yellow crystals, although the colony was still small. The "no man's land" between the mycelia was about 10 mm wide when strain 170 was combined with *Fomes annosus* and *Dothichiza populea* (Fig. 1 and 2) and 4—5 mm in combinations with other organisms. A similar trial was restarted with *Fomes annosus*, but now a myceliumblock of isolate 170 was placed in the center of the dish, whereas 4 blocks of *Fomes annosus* were spaced around it in the dish four days later. After a fortnight the growth of *Fomes* ceased.

These experiments demonstrated pronounced inhibition of growth of *F. annosus* in vitro and may indicate a possible approach to the control of that fungus in pine forests.

After growth of the other organisms had been inhibited, strain 170 continued to grow slowly towards them. Development of isolate 170 on plates with 2% wateragar was scanty; crystals were not seen at all. In liquid cultures mycelium growth is very excellent and mainly submerged. Malt solutions at no special concentration were used. At first crystals did not develop at all, though the fungus showed a very good growth. It was, however, possible to obtain a great amount of the yellow substance, when the solution was inoculated with a manifold quantity of mycelium. After 60 days the first crystals were obtained at the surface of the solution, gradually increasing and floating on the medium, accompanied by an increase in viscosity of the liquid. Maximum crystal formation in such cultures was reached after 80 to 100 days, at room temperature, but the author believes it depends largely on the amount of mycelium transferred to the solution. For practical purpose the agar method is preferable, since crystals were formed much more abundantly and in a shorter time.

It appeared possible to remove these crystals in a simple manner by means of a solution of chloroform, in which the substance dissolves very well.

The number of antibiotic substances known is very large. A great many have been isolated from Hyphomycetes and Actinomycetes. Little has been known, however, on such substances in the group of the Ascomycetes. WAKSMAN (1947) only mentions the genus *Chaetomium* (Pyrenomycetes) in which *Ch. cochliodes* forms an antibiotic substance designated as chaetomin.

Up till now antibiotic substances in the Discomycetes appear to have been very rarely reported, but this is probably due to the fact that this group has not been adequately investigated on these lines. The first important discovery in this field is of recent date and was described by WOOD (1953). This author found an antagonistic substance in the fungus *Lambertella corni-maris* Höhn. and performed some investigations on its antagonistic action against fungi and bacteria.

Its chemical nature was not studied, since the author mentions: "No attempt was made to isolate or identify these substances, . . .". Cultures of *Lambertella corni-maris* were received from the East Malling Research Station in England. After a series of transfers were executed, some crystals were observed. These crystals somewhat resemble those described in this paper, but are more orange-coloured. Probably the two substances are not identical, though this has still to be proved. The fungi are very distinct and not closely related. Further study on this substance from *L. corni-maris* is desirable, since it has antagonistic action towards *Botrytis cinerea*, many other fungi and bacteria.

TAXONOMY OF THE FUNGI.

Identification of these fungi is up till now very difficult, since the *Mollisioideae* are in need of a thorough revision. Although the present author has performed some starting-work in this group, the following names have been tentatively given.

1. ***Mollisia caesia*** Sacc. sensu Sydow, in Rehm's Krypt. Fl. 3, 516—517, 1896. non *Mollisia caesia* (Fuck.) Rehm, Krypt. Fl. 3, 516—517, 1896.

Description after 523: (Herbarium GREMMEN)

Apothecia 1.3—1.5 mm across, cream-coloured when fresh, light-yellow when dry; with a broad footstalk initiating in the substrate, which resembles a stroma. This stroma has a tissue consisting of colourless or lightbrown cells, partly prosenchymatic, partly parenchymatic. Excipulum with a darkbrown cortex consisting of roundish cells (textura globulosa). Hypothecium colourless, with large cavities, the structure resembles a textura intricata. Hymenium also colourless, 50—60 μ thick. Asci 57.0—60.8 \times 4 μ , J +. Ascospores 9.5—13.3 \times 1.9—2.5 μ , bacilliform, straight or somewhat curved, one-celled, seldom 2-celled. Paraphyses colourless, filiform.

Apothecia of this fungus were collected on dead, fallen twigs of *Salix* species, 14-IX-1950, Neerijnen (gem. Waardenburg), isolation 69a.

Cultures of this fungus developed a greyish mycelium on cherry decoction agar; on maltagar a grey to black one; mycelium growth was very slow.

Since the apothecia of this fungus seem to develop subepidermally, it would be a *Pyrenopeziza* sensu Fuck. emend. Nannf. The genus *Pyrenopeziza*, however, cannot be clearly delimited from *Mollisia* and is therefore rejected (GREMMEN, 1954). Hence these fungi are considered here to be true *Mollisia* species.

Another specimen was collected on dead branches of *Tilia* spec., 27-IX-1953, "De Dorschkamp", Wageningen, Gremmen 714, isolation 170.

Here the asci measure $49.4-57.0 \times 5.6-7.6 \mu$ and the ascospores $11.4-13.3-(15.2) \times 3.5-3.8 \mu$. In these cultures the characteristic crystals were developed too after a few days. The production in this strain was highest of all.

A culture about 6 weeks old showed blackish hyphae with a dense structure. After 6 months these structures developed into mature apothecia. In these fructifications mature asci measured $57 \times 4-5 \mu$, whereas the ascospores were $9.5-13.3-(15.2) \times 2.8 \mu$. The paraphyses were somewhat clavate and filled with a hyaline, oil-like substance, showing a greenish refraction of light.

2. *Mollisia fallens* (Karst.) Karst. Myc. Fenn. **1**, 191, 1871.

Trochila fallens Karst. Not. Sällsk. Fauna Fl. Fenn. **11**, 217, 1870.

Mollisia fallens (Karst.) Sacc. Syll. Fung. **8**, 332, 1889.

Description after 32: (Herbarium GREMMEN).

Apothecia 0.5—0.7 mm across, blackish-brown, with a yellow disc. A broad basal stroma-like part, 100—150 μ thick is present. It is partly prosenchymatic, partly parenchymatic. The excipulum is dark-brown, bending over the hymenial part. Its cortex bears rows of club-shaped brown cells, measuring 10—12 μ in length. After aging the characteristic basal part is unclear and the cortex of the exciple shows irregular, warty-like groups of cells, every cell representing a short clavus. In this stage the exciple measures 40—80 μ in thickness in the basal part, becoming thinner laterally. Hypothecium colourless, 40—80 μ thick. Hymenium colourless, about 60 μ thick. Asci (57.0)—60.8—68.4 \times 5.7 μ , J +. Ascospores

8.5—10.4 \times 1.9 μ , colourless, one-celled. Paraphyses colourless, filiform.

Apothecia were collected on dead branches of *Tilia* spec., 30-III-1951, "De Dorschkamp", Wageningen, Gremmen 32, isolation 71b.

This fungus agrees very well with the description of KARSTEN's *Mollisia fallens*. This was, moreover, verified with a drawing made by DENNIS from original material and checked with his description (DENNIS, 1950).

A c k n o w l e d g e m e n t s .

The author is very much indebted to the Director of the East Malling Research Station, Maidstone, Kent, for sending cultures of the fungus *Lambertella corni-maritima* Höhn. and to Dr R. W. G. DENNIS, The Herbarium, Kew, for his kindness in giving me some drawings of KARSTEN's original material, particular of *Mollisia fallens*.

S u m m a r y .

In this paper two species of *Mollisia*, viz., *Mollisia caesia* Sacc. sensu Sydow and *Mollisia fallens* Karst. are described. Both fungi inhabit dead twigs and form a yellow substance in vitro. It appeared that this substance was strongly antagonistic to some fungi, as shown by experiments with *Fomes annosus*, *Sclerotinia trifoliorum*, *Sclerotinia minor*, *Pollaccia radiosa*, *Dothichiza populea*, *Cladosporium* spec. and *Penicillium* spec. Growth of all these species was inhibited in the presence of strain 170. Production of the characteristic yellow substance (mollisine) was very high on maltagar, but was also obtained on cherry decoction agar, as well as in liquid cultures. A mycological description of both species has been given in this paper. Further publications on chemical and related properties of this substance will be published later by the institutes mentioned in the text.

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(The National Institute of Public Health, Utrecht).

PHAGE TYPING OF *SALMONELLA PARATYPHI* B; AN ARRANGEMENT OF PHAGE TYPES BASED ON THE STUDY OF LYSOGENICITY

by

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INTRODUCTION.

There are two groups of phage phenomena. The first group includes the phenomena in which bacteriophages lyse bacterial cultures; during this process the bacteriophages are reproduced and maintain their individuality, which is determined by plaque-form, lytic spectrum ¹⁾, thermolability and serology. This group of phenomena induced D'HÉRELLE to form the hypothesis that bacteriophage is an exogenic agent, a parasite that multiplies at the cost of the bacteria. However, a second group of phenomena was found from which those who regard bacteriophages as a bacterial product derive their arguments. This is spontaneous lysogenicity. OTTO and MUNTER (1921), LISBONNE and CARRÈRE (1922) and GILDEMEISTER and HERZBERG (1924) demonstrated that bacteria in pure culture are capable of producing bacteriophage.

At present two types of phage-producing strains are known (LWOFF, 1953), namely:

1. *Carrier strains*. These are considered to be a mixture of bacteria and bacteriophage. From these strains clones can be easily isolated which are sensitive to the phage, and which do not produce the phage.

2. *Lysogenic strains*. BORDET (1925) and BAIL (1925) and BURNET and Mc KIE (1929) plated lysogenic strains and found that every clone obtained by single-colony isolation produced the phage. This is characteristic for the lysogenic strains. Throughout

¹⁾ Following ANDERSON and FELIX (1953) the term "lytic spectrum" is used instead of the term "hostrange" throughout this paper.

this paper the phages derived from lysogenic strains are referred to as natural phages, following the terminology adopted by FELIX (see FELIX and CALLOW, 1951).

Since most of the separate colonies are derived from a single bacterium, it would appear that every bacterial individual contains at least one of these parasites, if indeed the bacteriophage is an exogenic agent, a parasite of the bacteria. Therefore, if a suspension of a known number of lysogenic bacteria is lysed by means of an agent that does not affect the natural bacteriophage, at least an equally great number of phage corpuscles should be liberated. BURNET and Mc KIE (1929) disrupted a suspension of lysogenic Gärtner bacteria with an unrelated extrinsic phage. However, the natural phage was not liberated. WOLLMAN and WOLLMAN (1938) dissolved a lysogenic *B. megatherium* strain with lysozyme. The natural phage was not liberated. The whole question was once more investigated by LWOFF and GUTMANN (1950), again with *B. megatherium*.

They studied washed single cells of a lysogenic strain of *B. megatherium* in a micro-drop, observed a number of divisions, and removed every second bacterium formed. After a series of 22 and 42 divisions not a single free phage-corpuscle had yet appeared, while the formed bacteria were still lysogenic. Thus the lysogenic *B. megatherium* transmits to the daughtercells the power to produce phage in the absence of demonstrable free phage. LWOFF and GUTMANN also observed how free phage-corpuscles are produced in the lysogenic strain. They watched microclones of *B. megatherium* for a reasonable length of time and noticed that the appearance of phage in the fluid is accompanied by the lysis of one of the bacteria. They also repeated WOLLMAN and WOLLMAN's experiments (1938) with lysozyme under the microscope. Thus lysogenic bacteria perpetuate hereditarily the power to produce bacteriophage. As most investigators refuse to accept the consequence of these experiments, namely, that the bacteriophage is a bacterial product, it was assumed that the bacteriophage is present in the bacterium in some latent form or other. This latent form is the prophage.

LEDERBERG and LEDERBERG (1953) and WOLLMAN (1953) showed in recombination experiments with variants of *E. coli* K 12 that the property to ferment galactose and the property to be lysogenic are always found together in the recombinants. This suggests that there

may exist a true genetic linkage between the character lysogeny and a bacterial gene, thus favouring the hypothesis that lysogeny has a nuclear determination.

LWOFF (1953) sums up the properties of the prophage as follows: Prophage like a gene is a specific structure endowed with genetic continuity. It is located at a specific chromosomal site; it is replicated in coördination with bacterial reproduction; it behaves like a chromosomal locus. Of a lysogenic bacterium LWOFF says that it possesses at a given site of a chromosome a specific genelike structure, the multiplication of which is coördinated with genic, chromosomal and bacterial reproduction.

Phage typing is a method that yields a varietal subdivision of bacterial species. The simplest procedure is to isolate a single bacteriophage for a given bacterial species, *e.g.*, from polluted surface water, and to test this phage on a great number of strains of this species. We thus find two types, the sensitive and the insensitive. With a second phage added to this system both types can be subdivided etc. Many typing systems have been based on this principle. This method will be referred to as the primitive method.

Phage typing attained significance when CRAIGIE and YEN (1938) showed that it is possible to obtain a varietal subdivision of *Salmonella typhi* by means of adapted preparations derived from a single phage. This system was extended by CRAIGIE and FELIX (1947). At present 41 types can be identified in this way. It need hardly be pointed out that this system is one of greater biological importance than the systems founded on the primitive method.

In epidemiological work the types are used as the "finger prints" of the bacteria. It was obvious to try to build up a similar system of phage types for *Salmonella paratyphi* B. FELIX and CALLOW (1943) isolated a bacteriophage which attacked certain strains of *Salm. paratyphi* B and could be adapted to a few other types. However, in the course of many years, only four adaptations of this bacteriophage were obtained. The system of FELIX and CALLOW, internationally used for phage typing, is based on these four adaptations and on five other phage preparations. (The biological value of these preparations is discussed in this paper). First a number of "types" was drawn up, and these had soon to be subdivided into "variations" (FELIX and CALLOW, 1951).

In the typing of the strains isolated in the Netherlands some

difficulties were encountered and therefore another principle was looked for, according to which *Salm. paratyphi* B could be subdivided into types. Spontaneous lysogenicity was thought of. It was known already that nearly all strains of *Salm. paratyphi* B are lysogenic (NICOLLE *et al.*, 1946, 1951; SCHOLTENS, 1950; FELIX and CALLOW, 1951). Considering the constant character of these phenomena, it seemed worth while to examine whether the spontaneously lysogenic strains of *Salm. paratyphi* B continued producing the same bacteriophage when passing from patient to patient, and from carrier to patient, whether "types" could be recognised by the identity of the natural phages of the bacteria, and in how far these types correspond with the types of the FELIX and CALLOW system. It was examined how far the identity of the natural phage of the bacterium could be used as a "fingerprint" of the infecting strain. In the course of this investigation it was found that there exists a parallellism between the lysogenic properties of the strains and their reactions to typing phage preparations and that the best way of defining types is to take both groups of properties into account. On the basis of lysogenic properties it appeared possible to arrive at a natural or phylogenetic system of phage types.

METHODS.

1. **Phage reactions.** The technique of CRAIGIE and FELIX (1947) and FELIX and CALLOW (1951) was followed throughout.
2. **Serological reactions.** The technique as described in a previous paper (SCHOLTENS, 1955) was utilised.
3. **The direct demonstration of the natural phages I, IV and VI.** A drop of broth culture of the strain to be investigated together with a drop of a fresh broth culture of the type strain 3a B62 of FELIX and CALLOW was seeded into 2 ml of broth and incubated for sixteen hours. The culture was heated in a waterbath of 56° C. during 15 minutes and examined in agar-plate tests on the following strains: B 228 (type 2 of FELIX and CALLOW), 1108 (type Midwoud) and 3a B62 (3a common type). The reactions of four of the natural phages are shown in Tabel 1. For serological confirmation and in order to show the presence of phage I in a mixture containing also phages VI and IV respectively, a parallel test is set up in which the strain is inoculated together with strain 3a B62 into broth to which either serum anti phage VI or serum anti

phage IV has been added in a dilution of 1/100. (The neutralizing titres of the sera used were 1/6400 or 1/12800). After 16 hours incubation the preparation is examined as described above and phage I alone is found or no phage at all can be demonstrated. Phage I can be recognised by the aspect of the plaques produced.

TABLE 1.

Routine demonstration of the natural phages I, IVb, IVa and VI.

Test strains		Reactions of the test strains to the natural phages			
strain	type	I	IVb	IVa	VI
B228	2 Felix and Callow	—	—	cl	cl
1108	Midwoud	—	cl	cl	cl
B62	3a Felix and Callow	cl	cl	cl	cl

Reactions to the directly-demonstrable, natural type-determining phages of the strains utilised in the examination for the presence of these phages in strains of *Salm. paratyphi* B.

Contaminating phages can mostly be recognised by the aspect of the plaques they produce. They may first be mistaken for phage IVa. Therefore, as a rule, serological confirmation is required, if the reactions of phage IVa are found.

Below it will be shown that it is sometimes the absence of certain natural phages, that is remarkable. It never happened that the presence of the bacteriophages I, IV or VI was demonstrated in a repeated and varied investigation (longer incubation, multiplication on other strains), if the first investigation according to this technique had given a negative result.

4. The direct demonstration of bacteriophage II.

A few drops of chloroform are added to 2 ml of a 24 hr. broth culture of the strain of type 1 of FELIX and CALLOW to be investigated and the culture is shaken vigorously. After settling for 1 hr. a few drops of the killed culture are added to 3 ml broth inoculated with strain 65 of *Salm. dublin* (SCHOLTENS, 1950). After 6 hr. incubation a drop of the grown culture is spotted on a lawn of bacteria of strain 65 *Salm. dublin*. (It must be emphasised that the bacteria in the broth culture are not killed). After 16 hr. incubation confluent

or nearly confluent pinpoint plaques of phage II may be seen. By performing the same experiment in broth to which serum anti phage II has been added the serological confirmation can be obtained.

THE SPONTANEOUS LYSOGENICITY OF *Salm. paratyphi* B.

Spontaneous lysogenicity can be shown in two different ways. The first method will be referred to as the direct method. In it a 24 hr. broth culture is filtered off and the filtrate is tested on a sensitive strain. On solid media plaques are found. It appeared that in strains of *Salm. paratyphi* B only a few different phages are found in this way. They occur singly, or in combination, in more than one of the phage types, as distinguished by phage reactions. These are the bacteriophages I, VI, IV and II, VII. These bacteriophages differ in lytic spectrum, plaque form, serology and thermostability. A characteristic feature of their lytic spectrum is that they do not attack the homologous strain.

The bacteriophages I, VI and IV influence the phage reactions and thus determine the type of the strains in which they are found. They have been called "phages type déterminants" by NICOLLE, HAMON and EDLINGER (1951). This term will be taken over and these phages will be referred to throughout as the type-determinating natural phages.

The bacteriophages II and VII produce small plaques. Bacteriophage VII is found in type 2 of FELIX and CALLOW (SCHOLTENS, 1950, 1952). The bacteriophage II can be isolated from types 1 of FELIX and CALLOW, Yersey, and Beccles-Meppel. Types 3b and 3a of FELIX and CALLOW also produce similar phages yielding small plaques (FELIX and CALLOW, 1951). It is possible that these phages play a part in the phenomena in the mixed cultures to be described below.

The natural phages do not attack the strain by which they are produced. An exception is observed in type Taunton of FELIX and CALLOW. This type excretes bacteriophage IV. This bacteriophage is met with in two varieties. The one attacks strains of a certain number of types and not the homologous type, the other variety attacks these strains and also the homologous type. Thus only strains of type Taunton are attacked by phage IVa, but not so by phage IVb. Strains of other types are either attacked by both variants or by neither of the two. The two phages IVb and IVa are

the typing bacteriophages Beccles and Taunton from the FELIX and CALLOW system.

The phages found in the strains by the direct method yield, when injected into the rabbit, very specific sera. These sera hardly react, or do not react at all, with the other bacteriophages found by the direct method.

However, there is also a second method by which further bacteriophages can be obtained from strains of *Salm. paratyphi* B (FLU, 1932). A bottle containing 60 ml of broth is inoculated with two strains of different types and incubated during a week. The culture is filtered and in the filtrate bacteriophages are found which are not met with in either of the strains by means of the direct method.

One of the strains may be substituted by its filtrate in which the other strain is incubated for a week. This longer incubation, however, is essential. It appears as if part of the process needs more time for its development. These bacteriophages differ from those found directly with respect to lytic spectrum, plaqueform and thermolability. They do react with sera that have been prepared with the phages found directly. They may react with the sera anti phage II and thus possess the serology II, or react with the sera anti phage VI and thus possess the serology VI.

THE NATURAL GROUPING OF PHAGE TYPES OF *Salm. paratyphi* B

The best method of explaining how, with the study of spontaneous lysogenicity, a natural grouping of phage types can be obtained is to present at once the result of this study. There are several series (groups) of phage types in which the types can be arranged in uniform manner according to their increasing content of the directly-demonstrable, type-determining, natural phages I, VI and IV. With this difference a difference in phage reactions runs parallel. In each series (group) a type is encountered that contains none of these three natural phages, followed, successively, by a type containing only phage I, a type containing only phage VI, a type containing the phages I and VI, and types that contain the phages I and IV, or IV only. For instance, in group A (Tables 2 and 3) the type 3b contains none of these three phages, the type "22" only the bacteriophage I, the type 3b variation 2 only the bacteriophage VI, type Dundee the two phages I and VI, and type Taunton (the Hague) only the bacteriophage IV. Group A is one of the most

complete groups. Up to the present not all of the corresponding types have been found in the other groups. The content of natural phage, or phages, influences the sensitivity of the bacteria to these phages (NICOLLE, HAMON and EDLINGER, 1951; NICOLLE and HAMON, 1951 „phages type déterminants”), so that the corresponding types show a corresponding pattern of phage reactions with these phages (see Tables 2 and 3).

TABLE 2
Natural grouping of phages

Groups of phage types				
A	M	S	J	B.M.
3 b (common type) “22”	3 b variation 1 Midwoud	“54” Sittard	“60” Jersey	Meppel
3 b variation 2 Dundee Taunton (the Hague)	B.A.O.R.	“18” “87”	3 a variation 3	

Survey of the phage reactions which the corresponding

The following rules and exceptions are observed. A strain that excretes a given bacteriophage is insensitive to it. The strains that excrete the bacteriophage VI are also insensitive to the bacteriophage IV. Reversely, the strains that excrete the bacteriophage IV are also insensitive to the bacteriophage VI. However, these strains (type Taunton of FELIX and CALLOW) are insensitive to the bacteriophage IVb but are sensitive to the bacteriophage IVa (see preceding section).

The inverse rule *i.e.*, that a strain of *Salm. paratyphi* B which is insensitive to a given natural phage may be assumed to produce this phage, is only partly applicable. Here the type 3aI (Schiedam) may be mentioned as an exception. This type contains only the natural type-determining phage I, but is nevertheless insensitive to phages IVa and IVb, and sensitive to phage VI. In this particular case the reactions to bacteriophages IV and VI, which usually run

parallel, do not coincide. On account of this remarkable combination of phage reactions these strains constitute a type.

Another exception are strains giving one of the patterns of phage reactions shown in Table 2, which fail to yield the expected natural phages (*e.g.*, strains that react like type Taunton, which do not produce bacteriophage IVb). However, these findings are so rare that they are not presented in the table as variants. They will be

types of *Salm. paratyphi* B.

B	Reaction to directly-demonstrable type-determining natural phages				Directly-demonstrable type-determining natural phages found in the types		
	I	IVb	IVa	VI			
3 a (common type)	cl	cl	cl	cl	—	—	—
3 a I variation 1, 2	—	cl	cl	cl	I	—	—
3 a I (Schiedam)	—	—	—	cl	I	—	—
3 a variation 4	cl	—	—	—	—	VI	—
3 a I (Leeuwarden)	—	—	—	—	I	VI	—
	—	—	cl	—	—	—	IV
Taunton (Kampen)	—	—	cl	—	I	—	IV

types of the groups (series) have in common.

discussed below in a separate paragraph.

The properties common to all the phage types belonging to the same group are: (a) the lysogenic properties which are manifested in mixed cultures; (b) the phage reactions with the bacteriophages generated in such mixed cultures. These phage reactions, which indicate groups of types, will therefore be called group reactions. Certain of the adapted preparations from the FELIX and CALLOW system also give group reactions.

Summing up, the types grouped horizontally in Table 2 thus have a corresponding content in directly-demonstrable natural phages and show a common pattern of reactions to these phages. The types grouped vertically have in common lysogenic properties which are revealed in mixed cultures and give the above mentioned group reactions. The phenomena are complicated in that the content of directly-demonstrable natural phages influences

TABLE
Natural groups of phage

Group	Types according to FELIX and CALLOW	Types found by the study of spontaneous lysogenicity	Phage group				
			Adaptations of phage 1 of FELIX and CALLOW				
			1	2	3a 3aI	Jersey	Beccles Meppel
			Nomenclature				
			1	2	3a	Jersey	
A	3b (common type)	"22"	—	—	—	—	—
	Beccles		—	—	—	—	—
	3b variation 2	"the Hague"	—	—	—	—	—
	Dundee		—	—	—	—	—
	Taunton		—	—	—	—	—
M	3b variation 1	"Midwoud"	—	—	—	—	—
	Beccles		—	—	—	—	—
	B.A.O.R.		—	—	—	—	—
S	3a	"54"	—	—	cl	—	—
	Beccles? 3a I?	"Sittard"	—	—	(cl)	—	—
		"18"	—	—	cl	—	—
	3a I (common type)	"87"	—	—	cl	—	—
J	3a, Jersey?	"60"	—	—	cl	cl	cl
	Jersey		—	—	(cl)	cl	cl
	3a variation 3		—	—	cl	cl	cl
B.M.	Beccles	"Meppel"	—	—	—	—	cl
B	3a (common type)	"Schiedam"	—	—	cl	—	—
	3a I variation 1, 2		—	—	cl	—	—
	3a I (common type)		—	—	cl	—	—
	3a variation 4	"Leeuwarden"	—	—	cl	—	—
	3a I (common type)		—	—	cl	—	—
	Taunton		—	—	(cl)	—	—

cl = confluent lysis

scl = semi confluent lysis

+1, +2, +3 increasing number of plaques

Survey of the natural grouping of a part of the phage types of *Salm. paratyphi* B. There are two kinds of phage reactions, first the group reactions which all types of one and the same group of phage types have in common, and second the type reactions which differentiate the types in the groups.

types of *Salm. paratyphi* B.

reactions										Directly demonstrable type determining na- tural phages found in the types		
reactions						type reactions						
Lytic spectra of the phages found in the mixed cultures						Directly demonstrable type determining natural phages						
e	d	c	f	h	b	I	IVb	IVa	VI			
of preparations in the system of FELIX and CALLOW												
Dun- dee						3b	Bec- cles	Taun- ton				
—	cl	+3	—	cl	—	cl	cl	cl	cl	—	—	—
—	cl	+2	—	cl	—	—	cl	cl	cl	I	—	—
—	cl	+1	—	cl	—	cl	—	—	—	—	VI	—
—	cl	—	—	cl	—	—	—	—	—	I	VI	—
—	cl	+2	—	cl	—	—	—	cl	—	—	—	IV
cl	—	cl	—	cl	—	cl	cl	cl	cl	—	—	—
cl	—	cl	—	cl	—	—	cl	cl	cl	I	—	—
cl	—	cl	—	cl	—	(cl)	—	—	—	—	VI	—
cl	cl	+2	cl	cl	+1	cl	cl	cl	cl	—	—	—
cl	cl	+3	cl	cl	+1	—	cl	cl	cl	I	—	—
cl	cl	+3	cl	cl	+1	cl	—	—	—	—	VI	—
cl	cl	+1	cl	cl	+1	—	—	—	—	I	VI	—
cl	cl	+3	—	scl	cl	cl	cl	cl	cl	—	—	—
cl	cl	+3	—	scl	cl	—	cl	cl	cl	I	—	—
cl	cl	—	—	scl	cl	cl	—	—	—	—	VI	—
cl	cl	+3	—	—	cl	—	cl	cl	cl	I	—	—
cl	cl	+3	—	cl	cl	cl	cl	cl	cl	—	—	—
cl	cl	—	—	cl	cl	—	—	—	cl	I	—	—
cl	cl	—	—	cl	cl	cl	—	—	—	—	VI	—
cl	cl	—	—	cl	cl	—	—	—	—	I	VI	—
cl	cl	—	—	cl	cl	—	—	cl	—	I	—	IV

m⁴ = plaques as dustlike particles, to be observed with the colony microscope
 (cl) = the reaction is not always, or even rarely present

Group reactions give: 1. the adaptations of phage I of FELIX and CALLOW and 2. bacteriophages found in the mixed cultures. Type reactions give the directly demonstrable, type determining natural phages I, IVa, IVb, and VI. These are determined by the presence of these phages in the strains. The type reactions of table 3 are the same as those of table 2.

the serological properties of the phages generated in mixed cultures.

LYSOGENIC PROPERTIES COMMON TO TYPES BELONGING TO THE SAME GROUP.

The lysogenic properties and phage reactions which are characteristic for the types of group A are the following. If a strain of one of the types of group A is inoculated into broth together with a strain of one of the types of group M and incubated for a week, a given mixture of bacteriophages is formed. In this mixture first bacteriophages are found, that attack all the types except those of group A. This lytic spectrum has been designated in Tables 3 and 5 as lytic spectrum e. In addition bacteriophages are found that attack all the types except those of group M. This lytic spectrum is designated d in Tables 3 and 5. It corresponds with the lytic spectrum e of the phage preparation Dundee in the system of FELIX and CALLOW. Thus the types of group A have in common the property of being resistant to the phages with lytic spectrum e, that attack all types except those of group A. They also have in common the property to form with strains of group M bacteriophages with lytic spectrum e.

Moreover in mixed cultures of strains of each of the types of group A with strains of each of the types belonging to one of the respective other groups, bacteriophages with a given characteristic lytic spectrum are found. For instance, with strains of types of group S bacteriophages are encountered with the lytic spectrum f, and in mixed cultures with strains of groups J or B bacteriophages with the lytic spectrum b are met with (Tables 3 and 4). This will be discussed with the groups concerned. Thus the types of group A have in common a group reaction (lytic spectrum e) and also the lysogenic properties that manifest themselves in mixed cultures.

In the same way the unity of the types of group M can be demonstrated. The strains of each of the types belonging to this group have a phage reaction in common, namely, they are not attacked by the bacteriophage which attacks all types except the strains of group M (lytic spectrum d). The lysogenic property they have in common is, that in mixed cultures with strains of group A they generate bacteriophages with lytic spectrum d, in addition to bacteriophages with lytic spectrum e. With the types of nearly all the

other groups, the types of group M again give bacteriophages with the lytic spectrum d, now combined with bacteriophages having a lytic spectrum that is nearly the image of lytic spectrum d, for they attack the types of group M to a higher titre than any of the other types. This lytic spectrum has been presented as lytic spectrum c in Tables 3 and 5. (This lytic spectrum is occasionally found with bacteriophages from the mixed cultures of types belonging to groups A and M, simultaneously with phages with the lytic spectra d or e).

Thus the types of group M have a characteristic phage reaction (lytic spectrum d) and particular lysogenic properties in common.

The unity of types belonging to each of the other groups is also based on the fact that bacteriophages with a given lytic spectrum are found in mixed cultures with strains of types belonging to other groups. Each group will be discussed separately. It is the lytic spectra of some of the bacteriophages formed that are characteristic; their serological properties depend on the content of directly-demonstrable natural phages of the strains that are grown in mixed cultures.

DEPENDENCE OF THE SEROLOGICAL PROPERTIES OF THE PHAGES FORMED IN MIXED CULTURES ON THE PRESENCE IN THE STRAINS OF THE DIRECTLY-DEMONSTRABLE NATURAL PHAGES VI AND IV.

In this section the mixed cultures of strains belonging to types of group A and M will be discussed. If the two strains grown in mixed culture both belong to types in which neither phage IV nor phage VI can be demonstrated directly, the bacteriophages found in the mixed culture possess small plaques and their serology corresponds with that of the directly-found phage II.

If one of the strains in the mixed culture, either that of group A or that of group M, or both belong to a type which produces spontaneously phage VI, then bacteriophages with larger plaques are found, simultaneously with small-plaque phages. The latter again show serology II; the phages with larger plaques react with sera anti phage VI up to the titre. Both serological forms are encountered with each lytic spectrum as follows:

- lytic spectrum e, serology II, small plaques
- lytic spectrum e, serology VI, larger plaques
- lytic spectrum d, serology II, small plaques
- lytic spectrum d, serology VI, larger plaques

Occasionally, the lytic spectrum c is also found, combined with each of the two serological forms (Table 4).

In a mixed culture of the strain Pty 4096, type 3 b variation 1 group M, and the strain Pty 10728, type Taunton (the Hague), group A, bacteriophages with lytic spectra d and e respectively and the serology IV were found, simultaneously with small plaque phages with the serology II. Phages with the following properties were found:

lytic spectrum e, serology II, small plaques
lytic spectrum e, serology IV, larger plaques
lytic spectrum d, serology II, small plaques
lytic spectrum d, serology IV, larger plaques

These phages mentioned above with larger plaques and with the serology VI and IV respectively have the propensity to split off small-plaque phages with the serology II, when they are regenerated on strain Pty 6450 (type Beccles, Capelle a/d Yssel). This is not observed when these phages are regenerated on strain Pty 3943 (type Sittard).

Table 4 gives a survey of the serological forms occurring in the mixed cultures of strains belonging to types of groups A and M. In the mixed cultures of strains from other groups of types parallel phenomena are found. In Table 4 the types of the groups A and M can be replaced by corresponding types from other groups.

From these observations it may be inferred that the phages from the mixed cultures are formed of elements from both strains, as sometimes a lytic spectrum that must be considered bound to an element from one of the strains, is found in combination with serological properties bound to an element from the other strain.

The lytic spectrum d is bound to an element from the strain of group M, for the bacteriophage with the lytic spectrum d bears the imprint of group M, since it attacks all strains except the strains from group M, in the same way as every natural phage attacks heterologous strains but not the homologous one. Moreover lytic spectrum d is always found when one of the strains in the mixed culture belongs to group M and is never found if no strain of this group is employed (Table 5). It may therefore be assumed, that this lytic spectrum is bound to an element from the strain of group M. In mixed cultures of strains from group M, which are devoid of

TABLE 4.

Dependence of the serological properties of the phages found in the mixed cultures on the content of the strains of directly-demonstrable natural phages.

Types of group M	Types of group A				
	Directly-demonstrable type-determining natural phages present in the types	3 b (common type)	"22"	3 b variation 2	Dundee
		Directly-demonstrable type-determining natural phages present in the types			
3 b variation 1	—, —, —	—, —, —	I, —, —	—, VI, —	I, VI, —
Midwound	I, —, —	s II	s II	s II or s VI	s II or s VI
B.A.O.R.	—, VI, —	s II	s II	s II or s VI	s II
		s II or s VI	s II or s VI	s II or s VI	s II or s VI

The serology of the phages found in the mixed cultures is indicated in the horizontal and vertical lines respectively.

s II serological properties similar to those of natural phage II.

s VI serological properties similar to those of natural phage VI.

phages VI en IV, with strains of various types of group A, phages are found with the lytic spectrum d and the serology VI, provided the strain from group A contains the directly-demonstrable bacteriophage VI (types 3 b variation 2 and Dundee); on the other hand, the lytic spectrum d combined with serology IV is found when the strain from group A contains the natural phage IV (type Taunton, the Hague). The serology of these phages is thus determined by the strain from group A. Since the lytic spectrum d derives from the strain of group M it is evident that elements from both strains occur in one and the same bacteriophage.

TABLE 5.

Survey of the lytic spectra of the bacteriophages found in mixed cultures of the types belonging to the groups indicated.

Group	Group				
	A	M	S	J	B.M.
M	(c), d, e				
S	f	c, d			
J	b	c, d			
B.M.	b	c, d, h			
B	b	c, d			
type 1 FELIX and CALLOW	f	c, d	g		

Lytic spectra of the phages found in mixed cultures of strains of the groups indicated in the horizontal and vertical lines, respectively. (The results with ungrouped type 1 of FELIX and CALLOW are also in the table.)

FURTHER GROUPS OF PHAGE TYPES.

Group S.

SCHOLTENS (1955) described a phage type Sittard which caused difficulties with the typing according to the system of FELIX and CALLOW. These strains reacted either as Beccles or as type 3a variation 1, that is to say, they gave irregular results with the phage preparations 3a and 3aI, on which the differential diagnosis between those two types in the system of FELIX and CALLOW depends. However, these strains have very characteristic lysogenic properties. They produce directly only the bacteriophage I; in mixed cultures

with strains of group A they produce a bacteriophage which attacks strains of types 1 and 2 of FELIX and CALLOW, the strains of type Sittard and also some untypable strains (lytic spectrum f, Table 3). By means of this fairly specific phage reactions the strains of type Sittard can be recognised and differentiated from strains of other subgroups of types Beccles and 3aI.

In addition, type Sittard strains produce in mixed cultures with strains of type 1 of FELIX and CALLOW a very remarkable bacteriophage. The latter called phage V in a previous publication (SCHOLTENS, 1950) is very thermolabile. It is inactivated already at 56°C. and it differs serologically from the bacteriophages found by the direct method.

Other strains were found which reacted also with the bacteriophages with the lytic spectrum f, but which differed from type Sittard with regard to their content of directly-demonstrable, type-determining, natural phage and with regard to the reactions to these phages. First a strain Pty 3554 (type "54") was encountered in which neither phage I, nor VI or IV, could be demonstrated directly; later a strain Pty 818, type "18" was identified in which only phage VI was present, and a strain 14487 (type "87") in which the phages I and VI could be demonstrated. Of these last two types more than one strain were found.

Grown with group A strains they formed, amongst others, bacteriophages with the lytic spectrum f. The presence in the strains of the directly-demonstrable phage VI made the phenomena more complicated, so that very complex mixtures of phages were observed. These strains therefore share with type Sittard lysogenic properties which manifest themselves in mixed cultures. In this way a group of types could be drawn up, group S in Tables 2 and 3, analogous to groups A and M.

Group J.

Type Jersey is one of the types of the system of FELIX and CALLOW that is identified by means of an adapted phage preparation. However, this preparation also attacks another type of this system (3a variation 3) and also a strain Pty 4860, isolated at the National Institute of Public Health, which differs from the other two types. According to their content of directly-demonstrable natural phages, and according to their reactions with these phages, the three different types can be arranged in the same way as the types in groups A,

M and S (Tables 2 and 3). The types of this group J differ from each other in their reactions to the directly-demonstrable natural phages. They react in identical manner to the phage preparations that give group reactions. Here the remarkable fact appears that one of the adaptations of phage 1 employed in the system of FELIX and CALLOW gives group reactions (the preparations 3a and 3aI of this system lyse strains belonging to more than one of the groups (Table 3)).

In mixed cultures strains of the types from group J produce phages with a characteristic lytic spectrum as shown in Tables 3 and 5. These observations enable us to arrange these types in the way described above. The presence of directly-demonstrable natural phage VI in strains from group J plays the same part in the serological phenomena in mixed culture as that indicated above for the other groups.

Group B. M.

The strains of type Beccles (Meppel) described by SCHOLTENS (1952a) are provisionally arranged as a single type in a separate group. It appeared that phage 1 of FELIX and CALLOW, when adapted to type Jersey, acquires a greater affinity to certain strains of the group Beccles of this system. This is not manifested in the critical test concentration, only in greater concentrations. Starting from the original adapted preparation Jersey, an adapted preparation could be obtained which not only attacks type Jersey but also type Beccles (Meppel) up to the critical test concentration. Since it has been shown above that adapted preparations of phage 1 of FELIX and CALLOW give group-reactions, there is good reason for placing this type in a separate group provisionally. Moreover, a further phage reaction, peculiar for this type, was found, which can rightly be regarded as a group reaction. In mixed cultures of strains of type Beccles (Meppel) with strains of type B.A.O.R. (group M), bacteriophages were found which attack all the types, except the type Beccles (Meppel) [(lytic spectrum h); see Tables 3 and 5]. Type Jersey may be attacked somewhat less strongly than other types. This phage reaction is comparable with those of phages with the lytic spectra d or e, also isolated from mixed cultures with strains of group M, which also attack all types except those of a given group. Since these latter phages give group reactions, the reactions of bacteriophages with the lytic spectrum h may also be looked upon as a group reaction.

SCHOLTENS (1952a) described as a subtype of Beccles, strains, which in all phage reactions corresponded with the type Beccles (Meppel), but which did not excrete phage I. These strains are discussed below as type Beccles (Capelle a/d Yssel).

There are further relationships between the types Beccles (Meppel) and Jersey, in addition to those manifested by sensitivity to typing phage preparations (see above and Table 3). The directly-demonstrable phage II, found in type Jersey, is also met with in type Beccles (Meppel). Moreover, both belong to those few types that ferment rhamnose slowly, as will be discussed later.

Group B.

In discussing the foregoing groups it was described that a member of bacteriophages give group reactions. These are the phages with the lytic spectra c, d, e, f and h, formed in mixed cultures, and certain of the adapted preparations of phage 1 of FELIX and CALLOW. These preparations give more or less specific group reactions with types of given groups. With the types belonging to other groups they give, in fact, no specific reactions, but all the types of a given group react, as a rule, in uniform manner.

Six of the types so far ungrouped give identical reactions to the phages with the above mentioned lytic spectra, and those types may also be arranged according to their increasing content of natural phages I, VI and IV. These are the types 3a (common type), 3aI variation 1, 2, 3aI (Schiedam), 3aI Leeuwarden and Taunton (Kampen) (see Tables 2 and 3). This grouping is supported by the behaviour of the above-mentioned types in mixed cultures; with strains of types of group A they produce bacteriophages which attack the strains of the types from groups J, B. M. and B (lytic spectrum b) (Tables 3 and 5). Phages with this lytic spectrum are also found in mixed cultures of strains from groups J and A, and groups B.M. and A. This phage reaction may be added to those mentioned above. Nevertheless group B appears to have the character of a residual group, from which other groups may perhaps be split off in the future.

TRANSFORMABILITY OF TYPES.

The existence of series of phage types has already been demonstrated by NICOLLE and HAMON (1951), HAMON and NICOLLE (1951)

and NICOLLE, HAMON and EDLINGER (1951). They tried to transform the types which are less rich in natural phages into types which are richer in them, and they succeeded in preparing the latter types from the former. NICOLLE and his co-workers found that there are a number of basic types (3a, 3b and type 1 of FELIX and CALLOW) from which certain other types could be obtained and no others. They considered these basic types as "pères de familles de types" and originally thought these types were devoid of natural phages. However, FELIX and CALLOW (1951) demonstrated the presence of natural phages in all these strains. There is clearly a connection between the phenomena discovered by NICOLLE and his co-workers and those described here.

It is easy to transform the types which possess neither phage I, nor phages VI, or IV into the related types that contain the phage I. This was observed in nearly all series of phage types also with types 3a common type and with 3a I variations 1, 2.

RHAMNOSE FERMENTATION AND GROUPS OF PHAGE TYPES.

In *Salm. paratyphi* B strains occur that ferment rhamnose readily and others that ferment rhamnose slowly (KRISTENSEN and BOJLEN, 1929). All strains of groups J and B.M. are slow rhamnose fermenters. Also the few strains of type 2 found in the Netherlands and many strains of type 1 (common type) are slow rhamnose fermenters. All these are types for which specific adaptations of bacteriophage 1 of FELIX and CALLOW exist. Slow fermentation of rhamnose apparently is a property which all the types belonging to certain groups of phage types have in common.

THE DIRECT DEMONSTRATION OF NATURAL PHAGES IN STRAINS OF *Salmonella paratyphi* B.

A strain containing certain natural phages is resistant to certain phages. On the other hand with *Salm. paratyphi* B, a given pattern of resistance is not always accompanied by the presence of a given natural phage. When the types are considered to be defined by the lytic reactions, produced by the typing phages we may say that the natural phages characteristic of the phage types may occasionally be absent. For instance, strains of type 3aI Leeuwarden were found which did not contain the natural phage I; other strains of this type did not contain phage VI; and strains of type Taunton were en-

countered, which had no natural phage I or no natural phage IV.

In this connection the few strains found of type Taunton (the Hague) may be mentioned; they did not contain the natural phage I and did not react to the critical test concentration of this phage (*i.e.*, phage 3b of FELIX and CALLOW). Also the strains of type Beccles (Capelle a/d Yssel) described in previous papers (SCHOLTENS 1950, 1952, 1955), can be regarded as strains of type Beccles (Meppel) in which the natural phage I is lacking.

Strains in which the presence of natural phage I cannot be demonstrated and which do not react to the critical test concentration of this phage, sometimes give lysis when tested with $\times 1000$ the critical test concentration. They may however, be even more resistant, so that with this test too, they may not be identifiable with certainty. The identification of such strains requires an investigation into the content of directly-demonstrable phages as described above. Such variants are probably of some epidemiological significance; some outbreaks of type Beccles (Capelle a/d Yssel) were observed. However, because of their rare occurrence, the detailed investigation of these strains could be omitted in practice.

In certain special cases the direct demonstration of natural phages was significant. By demonstrating the presence of bacteriophages I and IV in some strains which otherwise showed no phage reactions whatever, it could be made probable that the strains belonged to type Taunton.

Another instance of the practical application of the direct demonstration of the natural phage is the following. A nurse fell ill, showing atypical clinical symptoms. Bacteria of type Taunton were isolated from her faeces. However, a few years earlier she had suffered from paratyphoid-B fever and the question arose whether she was a chronic carrier. The strain isolated during her first illness was untypable and produced the natural phage VI, whereas the type Taunton strain produced phages I and IV. A re-infection with another type became therefore probable.

Type 1 of FELIX and CALLOW (common type) produces the natural phage II (SCHOLTENS, 1950). The latter can be easily demonstrated in the way described above. In an outbreak at Eindhoven in 1949, however, strains of type 1 of FELIX and CALLOW (common type) were found, in which the presence of phage II could not be shown in this way. These strains were d-tartrate negative. Some of the chronic carriers remaining from the 1949 outbreak continued to

excrete the abnormal strains; therefore this fact is probably of epidemiological significance.

POSSIBILITIES OF EXTENDING THE SYSTEM AND OF ITS PRACTICAL APPLICATION.

As a result of the foregoing observations a number of questions of theoretical and practical interest offer themselves.

(a) Tables 2 and 3 show that several of the groups postulated are still incomplete. The missing types may yet be found.

(b) It is evident that further series of types will have to be drawn up. Two of the main types of the FELIX and CALLOW system have not yet been included in Tables 2 and 3, namely type 1 and its "variations" and type 2. The latter might be the single representative so far found of a further group.

(c) Some of the groups shown in the tables may be found to be divisible by a new group reaction.

(d) It is possible that further phage types of *Salm. paratyphi* B exist in other parts of the world, which cannot be classified under a similar series.

Obviously the task of extending the suggested natural system and the question of the eventual application of these findings to the routine typing of *Salm. paratyphi* B require further research on a collaborative international basis. The main findings reported in this paper have been communicated to the International Committee for Enteric Phage Typing and a co-operative investigation by members of one of its special subcommittees has been initiated (Report of Proceedings, 1955).

THEORETICAL CONSIDERATIONS.

In considering whether a certain structure in an organism is part of this organism, we should not raise the question whether in our opinion this structure is noxious to this organism, or whether we find that something of this nature cannot be part of this organism, but we must investigate whether this structure is found in each individual of this species. Considering the occurrence of spontaneous lysogenicity in *Salm. paratyphi* B we see, that, as a matter of fact, not every strain of *Salm. paratyphi* B contains one and the same natural phage. However, every strain of a given phage type, possesses the same phage, or the same combination of phages. There-

fore we may regard the phage types, or perhaps the series of phage types, as the smallest units in the system of Linneaus, in which there would be a functional difference between the phage types. The fact that all the types of group J ferment rhamnose slowly and thus have a second hereditary property in common, is in favour of this view. The fact that the types of each of the series can be transformed into one another is also in support of this opinion.

A structure present in each individual of a given species does not represent a "pathological" abnormality, but the normal. The prophage, a genelike structure in the bacterial chromosome, might be a gene of the bacterium. In our opinion the facts described here and those found by other workers during the past few years, argue for the opinion that the bacteriophage is a bacterial product. A similar conclusion was arrived at by FELIX (1947, 1952).

Summary and conclusions.

1. A natural system of phage types of *Salm. paratyphi* B has been described.

2. Each group comprises a number of different phage types arranged according to the presence in the strains of the three natural phages I, IV and VI.

3. The phage types belonging to each of the groups so far postulated have two characteristics in common:

(a) all give identical lytic reactions (group reactions) to those typing phages that are specific adaptations of phage 1 of FELIX and CALLOW, and to bacteriophages found in mixed cultures;

(b) all elaborate identical phages when grown in mixed cultures with strains belonging to one of the other phage types of a different group.

4. The subdivision of groups into types is based on the lytic reactions to phage preparations representing the natural phages I, IVa, IVb and VI (type reactions). The first three of these phages correspond, respectively, to the typing phages 3b, Taunton and Beccles of the FELIX and CALLOW scheme.

5. The possibilities of extending the natural system and of its practical application are indicated.

6. The bearing of these findings on the problem of the nature of bacteriophage is discussed.

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THE EFFECT OF ANTIVACCINIA GAMMA GLOBULIN ON SMALLPOX VACCINATION IN VIEW OF A PROPOSED ATTEMPT TO PREVENT POSTVACCINAL ENCEPHALITIS¹⁾

by

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INTRODUCTION.

Formerly passive immunization against smallpox and vaccinia was a more frequent subject of investigation than it is nowadays. As early as 1895 HLAVA and HONL showed a complete or nearly complete protection against vaccinia in school children after a prophylactic injection of 3 to 10 ml of immune calf serum. Protective antibodies could be demonstrated in sera of man and calves (STERNBERG, 1896; BÉCLÈRE, CHAMBON and MÉNARD, 1896, a.o.), and in sera of rabbits (SATO, 1921, a.o.) and horses (LEDINGHAM, MORGAN and PETRIE, 1931). Natural passive transmission of protective antibodies and vaccinal immunity from mothers to their foetus or infants was shown in man (BÉCLÈRE, CHAMBON and MÉNARD, 1896), rabbits (SATO, 1921; ANDERSEN, 1937) and swine (NELSON, 1932).

Most of the work on passive immunization was carried out in rabbits. Intravenous injection of immune rabbit serum protects the rabbit skin from vaccinia infection (GASTINEL, 1913). Successful passive immunization depends upon the amount of serum injected (HUNT and FALK, 1927), though comparatively small doses (3 ml) are still effective (ANDREWES, 1929). Serum injections given before the virus inoculation are far more protective than those given afterwards (ANDREWES, 1929; GREEN and PARKER, 1942). The effect of

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the passive immunization remains on the same level even if the infection is delayed until 5 days (VIEUCHANGE, 1939a) or 7 days after the serum injection (ANDERSEN, 1937a). If, however, the injection of serum follows vaccinia infection, the interval between the two can be prolonged no more than 24 hours, without loss of the protective activity (VIEUCHANGE, 1939b).

ANDERSEN (1937a) could demonstrate virus neutralizing antibodies in the blood of rabbits until 7 days after their passive immunization.

It is a remarkable fact that vaccination reactions occurring after the injection of large quantities of virus into passively immunized rabbits are delayed, whereas those in actively immunized rabbits are accelerated (ANDERSEN, 1937a). An important feature of vaccination with simultaneous passive immunization is the development of active immunity even in a good many rabbits (55%) in which vaccinia reactions were completely prevented by the serum injection (ANDREWES, 1929).

Part of the earlier work has lost interest. The effective prevention of smallpox by well organized mass vaccinations has diminished the interest in the passive side of smallpox immunization. Moreover, the resulting enhancement of hypersensitivity to animal antigens has largely restricted the application of immune animal serum. Replacing the animal sera by human serum means introducing another and even graver danger caused by serum hepatitis infection.

More recently, since it has proved possible to transfer human antibodies safely to man by means of the gamma globulin fraction of the serum, passive immunization against virus diseases, such as measles, infectious hepatitis and poliomyelitis, has gained new interest. In the field of smallpox vaccination VAN BOUWDIJK BASTIAANSE (1947; quoted by SPAANDER and HOORWEG, 1953) proposed that antivaccinia gamma globulin should be given a trial in the prevention of postvaccinal encephalitis. As a matter of fact, the frequency of encephalitis complications after revaccination is only a small fraction of that after vaccination. Vaccinia neutralizing antibodies were first demonstrated in human gamma globulin by JANEWAY (ENDERS, 1944). VERLINDE and SPAANDER (1949) found neutralizing antibodies in vaccinia convalescent gamma globulin derived from a group of recently vaccinated persons. The neutralization index of vaccinia convalescent gamma globulin can be de-

terminated by a modified neutralization technique in duck eggs (GISPEN, 1953); it was found to be 10^3 , that is a ten-fold of that found usually in non-fractionated immune sera of man and rabbits. Now that the inoculation of gamma globulin has been considered as a measure for the prevention of postvaccinal encephalitis, one may ask whether neutralizing antibodies can be found in the serum of persons inoculated with reasonable quantities of antivaccinia gamma globulin. And if so, how long are they demonstrable *in vivo*? One should also know the minimum quantity of gamma globulin required for intramuscular injection to lend virulicide properties to the blood, or, perhaps to raise a barrier against viraemia.

Moreover, it is not known, whether the gamma globulin effects the primary vaccination reaction, or interferes with the active formation of neutralizing antibodies. Therefore further investigations into the effect of passive immunization on the antibody titer and vaccination reactions of man seem to be justified.

METHODS.

A batch of lyophilized vaccinia convalescent gamma globulin, obtained from the Central Laboratory of the Blood Transfusion Service, Amsterdam, was used during the experiment. The neutralization index of an 8 per cent solution of the gamma globulin was 10^3 in repeated virus neutralization tests.

The neutralization index was determined with a constant quantity of the 8 per cent gamma globulin solution or serum and a series of 10-fold dilutions of a vaccinia elementary bodies suspension on the chorioallantois of duck embryos. The sera and gamma globulin were heated at 60°C . for 30 minutes in order to eliminate non-specific thermolabile antivaccinia activity. Readings of the infection of membranes were made according to the presence of confluent reactions on at least one third of the chorioallantoic membrane, separate foci being ignored. Details of this neutralization technique were described elsewhere (GISPEN, 1953). In addition it must be stressed here that the elementary bodies suspension always had the same infection titer (10^{-5}). The reactions on the chorioallantoic membranes were inspected through the shell opening with the aid of a mirror-lamp attached to the forehead. The neutralization index was calculated from the ratio $\frac{\text{infection titer of virus} + \text{saline}}{\text{infection titer of virus} + \text{antibodies}}$.

Smallpox vaccinations were carried out with glycerinated calf

lymph from the Amsterdam Vaccine Lymph Establishment. Two scarifications of 5 mm each were made in the deltoid region. Combined passive-active immunizations were carried out by intramuscular inoculation of the antivaccinia gamma globulin in the gluteal region and smallpox vaccination 0—3 days afterwards. As ampules containing 1 ml of 16 per cent gamma globulin are generally used now, the doses mentioned below have been converted to a 16 per cent concentration, so that 1 ml indicates the quantity of 1 ampul (160 mg of dry gamma globulin).

Passive transfer of vaccinia neutralizing antibodies by means of gamma globulin.

The transfer of neutralizing antibodies was studied first in two adults (A and B), who had not been vaccinated before and whose blood serum did not show neutralization. They were inoculated intramuscularly with 6 and 12 ml of gamma globulin respectively. Next day their blood was examined for vaccinia neutralizing antibodies and this was done again one day after the inoculation and on several occasions during the next few weeks. The results given in table 1 show that neutralizing properties could be transferred in this way and remained demonstrable in the blood of the recipients for 2 to 3 weeks.

TABLE 1.

Period during which vaccinia neutralizing antibodies circulated in the blood of two persons (A and B) after intramuscular inoculation of antivaccinia gamma globulin.

Days	Log Neutralization index of the sera	
	A inoculated with 6 ml of the gamma globulin	B inoculated with 12 ml of the gamma globulin
0	0	0
1	(inoculation)	(inoculation)
2	1	1
7	1	1
14	1	1
21	0	1
28	0	0
35		0

After this, 44 non-vaccinated adult emigrants were inoculated intramuscularly with antivaccinia gamma globulin. Seventeen of them received 2 or 3 ml, whereas 27 received 6 ml or 12 ml of the gamma globulin. Two blood samples were drawn from each of them: one sample just before the gamma globulin injection and another two days afterwards. The neutralization index of each sample was determined in duck eggs. The results are shown in table 2 and figures 1—2.

TABLE 2.

The effect of various quantities of antivaccinia gamma globulin on the neutralization index of the blood, two days after intramuscular inoculation.

Quantity of gamma globulin	Number of persons inoculated	Number of inoculated persons showing an increase of neutralization index		
		10-fold	100-fold	total
2 ml	11	0	0	} 0
3 ml	6	0	0	
6 ml	24	15	3	} 21
12 ml	3	3	0	

The frequency with which neutralizing antibodies were found before the artificial immunization was rather high: sera from 9 out of the 44 non-vaccinated persons showed this activity (see fig. 1). One of the positive sera showed an index of 10^2 , the index of the others being 10. It may be assumed that natural contact with cowpox or vaccinia in this country is not rare. In addition it has recently been shown that a vaccinia neutralizing factor, which is thermostable at 58°C ., may be found in sera from groups of non-vaccinated infants at the age of 3 1/3 to 6 months old (DOORSCHODT, 1954).

None of the 17 persons who received 2 or 3 ml of the gamma globulin showed an increase of the neutralization index, whereas 15 out of 24 persons inoculated with 6 ml and the 3 inoculated with 12 ml did. There is a significant difference between the 2 to 3 ml group and the 6 to 12 ml group in this respect (see table 2 and fig. 1—2). Therefore, 6 ml of the gamma globulin was the minimum dose required to demonstrate an increase of neutralizing antibodies in the serum of inoculated adults.

Theoretically, one may expect about a 4-fold increase of the neutralization index in the 6 ml group. So the 10-fold increase which

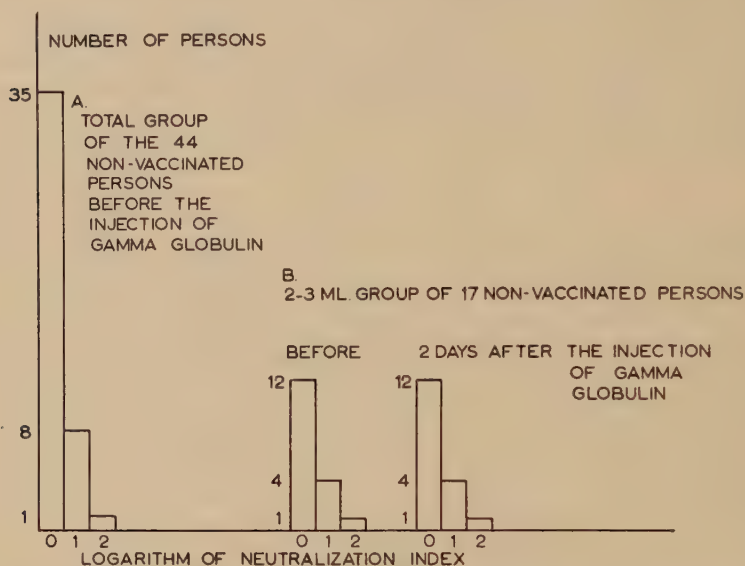


Fig. 1. Frequency of vaccinia neutralization indices in: (A) 44 non-vaccinated adults, (B) 17 non-vaccinated adults before and 2 days after the inoculation of 2 or 3 ml of antivaccinia gamma globulin.

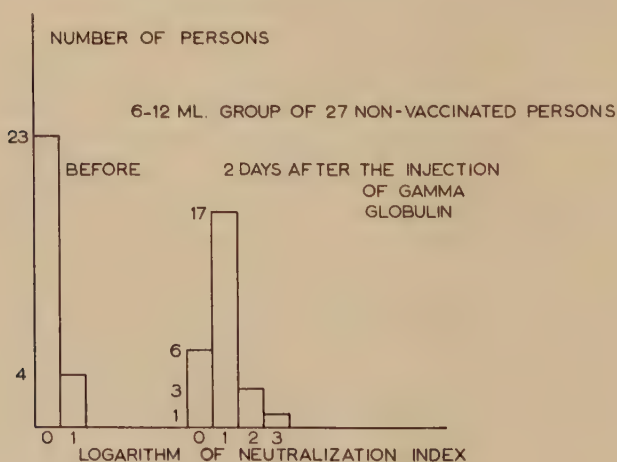


Fig. 2. Frequency of vaccinia neutralization indices in 27 non-vaccinated adults before and 2 days after the injection of 6—12 ml of antivaccinia gamma globulin.

was actually found in 15 out of 24 persons, is not far beyond expectation. In view of the method of measuring the neutralizing effect of sera, the cases which showed a 10-fold increase of neutralization index, might have had an antibody increase which is less than a 10-fold.

The effect of the antivaccinia gamma globulin on vaccination reactions.

If antivaccinia gamma globulin is given at the time of vaccination, the possibility of repercussion on the development of a vaccinal reaction must be considered. If large quantities of antibodies are transferred passively, the normal primary reaction upon vaccination, *i.e.*, multiplication of the virus, might be suppressed and, therefore, the active part of the immunization might fail.

The antivaccinia gamma globulin was tested in various doses (1, 2—3, 6 and 12 ml) as to the effect on primary vaccination reactions in a group of 1010 non-vaccinated persons over the age of 16. The vaccinations were carried out 0—2 days after intramuscular inoculation of the gamma globulin. The reactions were classified according to various types. The results are given in table 3. Column I of the table shows the number of non-reactors in various groups. There is no evidence that the percentage of non-reactors increases

TABLE 3.

Frequency of types of reaction; smallpox vaccination subsequent to intramuscular inoculation of antivaccinia gamma globulin within 0—2 days.

Quantity of gamma globulin injected	Number of persons treated	Types of reaction after primary vaccination				
		I no reaction ¹⁾	II primary	III accel- erated	IV delayed and abortive	III+IV modified
1 ml	403	6	393	4	—	4(1%)
2—3 ml	549 ²⁾	1	543	5	—	5(0.9%)
6 ml	55	7	48	0	—	0
12 ml	3	0	2	0	1	1
Total	1010	14	986	9	1	10(1%)

¹⁾ It cannot be decided whether the absence of vaccinal reaction in these cases is due to passive immunization or to vaccination technique.

²⁾ Only 10 of these persons were treated with 3 ml; each of the others received 2 ml.

according to the quantity of gamma globulin injected. The exact cause of this non-reacting remains obscure as the vaccination technique might also have been of influence. Anyhow, a second vaccination of the non-reactors after one week, without other gamma globulin injections, was successful, but for one person who did not react before the third vaccination attempt, and two others who did not react even after three repeated vaccinations at intervals of one week each. The non-reactors were therefore left out of consideration.

Table 3 shows equal percentages of accelerated reactions in the 1 ml and 2 to 3 ml groups. It may be worth mentioning that even 6 ml of the gamma globulin, which lent a definite virucide power

TABLE 4.

Development of vaccinal reaction, formation of vaccinia neutralizing antibodies and immunity against revaccination in 3 susceptible adults; smallpox vaccination 2 days after the intramuscular inoculation of a large quantity of antivaccinia gamma globulin (12 ml).

Items	Immunized persons		
	I	II	III
Maximal reaction	17th-20th day	8th-9th day	8th-9th day
Largest diameter of areola	20 mm	100 mm	92 mm
Type of reaction	delayed, abortive	primary	primary
Neutralizing antibodies ¹⁾ before immunization	0	0	0
2-3 days after imm.	1	1	1
1 week " "	1	1	1
2 weeks " "	1	1	1
3 weeks " "	1	1	1
4 weeks " "	0	1	1
6 weeks " "	0	1	1
1st revaccination after	2 ¹ / ₃ months	10 months	7 ¹ / ₂ months
Maximal reaction after			
1st revaccination between.	9th-10th day	1st-3rd day	1st-3rd day
Type of reaction after			
1st revaccination	primary 120 mm	„immediate”	„immediate”
Neutralizing antibodies ¹⁾			
5 weeks after 1st revaccin.	1		
2nd revaccination after	1 year		
Type of reaction after			
2nd revaccination	„immediate”		

¹⁾ Expressed in logarithm of neutralization index.

to the blood of 18 out of 24 persons (table 2) did not change the vaccinal reaction more frequently than 1 ml did. So it is not possible to attribute the accelerated reaction which was seen in 1 per cent all cases to immunological modification by passively transferred antibodies nor to an allergy transmitted by the gamma globulin. This acceleration is more likely to be connected with the neutralizing antibodies in sera of non-vaccinated persons, referred to above.

As the quantities of gamma globulin which we used might be too low to modify the reactions, 12 ml of the gamma globulin were injected into 3 serologically non-immune adults, 2 days prior to their smallpox vaccination. The effect of this combined passive-active immunization is shown in tables 3 and 4. The vaccination was successful, *i.e.*, followed by a primary vesicular reaction, in 2 of the persons treated (II and III). One of them (I) showed a delayed abortive non-vesicular papular response between the 17th and 20th day after the vaccination. Such modified reactions might be the rule with still higher doses of the gamma globulin, but shortage of gamma globulin did not allow of further trials with impracticably high doses.

The effect of the antivaccinia gamma globulin on active antibody formation.

The immunological response to vaccination depends on the development of a vesicle. Nevertheless, one might ask whether moderate quantities of the gamma globulin, which do not inhibit the vesicular reaction, would interfere with the formation of neutralizing antibodies.

Eleven fully susceptible persons were vaccinated one day after an intramuscular injection of 2 ml of the gamma globulin. The neutralization index of their blood was determined (1) just before the injection, (2) at the time of vaccination and (3) two weeks later. Ten others, who were treated in the same way, were examined for neutralizing antibodies 6 weeks after the vaccination. All persons showed a primary reaction. The neutralization indices are shown by table 5. They indicate that neutralizing antibodies were formed within 2 weeks after the vaccination. In only one person no increase of the neutralization index could be demonstrated.

Moreover, table 4 shows that even as much as 12 ml of the gamma globulin solution did not inhibit the active formation of antibodies

TABLE 5.

Development of neutralizing antibodies after combined active-passive immunization against vaccinia; smallpox vaccination: one day after the intramuscular inoculation of 2 ml of antivaccinia gamma globulin.

Case No	Logarithm of neutralization index			Case No	Log. of neutralization index 6 weeks after vaccination + g. globulin 2 ml	Case No	Log. of neutralization index of re-vaccinated persons, 1 to 6 months after revaccination
	Before inoculation	1 day after inoculation of g. globulin	2 weeks after vaccination + g. globulin				
1	0	0	1	12	1	22	2
2	0	0	1	13	2	23	1
3	1	1	1	14	2	24	2
4	1	1	2	15	1	25	0
5	0	0	1	16	1	26	2
6	1	1	2	17	3	27	0
7	0	0	1	18	2	28	0
8	0	0	1	19	2	29	1
9	0	0	2	20	2	30	2
10	0	0	1	21	1	31	2
11	0	0	2			32	2

in two persons whose subsequent vaccination was successful. Anyhow, the neutralizing activity of their sera did not show the rapid decrease which is always seen in case of exclusively passive immunization. Another, who was injected with an equal dose, had no vesicular reaction and therefore lacked the stimulus for antibody formation, so that the passively transferred neutralizing antibodies disappeared from the blood after 3 weeks (table 4).

The effect of the antivaccinia gamma globulin on the development of immunity to revaccination.

As the immunizations were carried out in emigrants, it was but casually possible to follow up the development of immunity to revaccination. Revaccination could be accomplished in 121 persons, who were vaccinated successfully 0—2 days after inoculation with 2 ml of the gamma globulin. The reactions upon the revaccination, which are given in table 6, were invariably classified as "immediate", the lapse of time since vaccination being 6—14 months. This indi-

TABLE 6.

Development of immunity against revaccination in 124 persons after combined active-passive immunization; smallpox vaccination 0 — 2 days after the intramuscular inoculation of antivaccinia gamma globulin.

Number of treated persons	Dose of gamma globulin	Reaction after vaccination	First revaccination			
			Interval in months between vaccination and revaccination	Reactions classified as		
				Immediate	Accelerated	Primary
113	2 ml	primary	6	113	—	—
1	2 ml	primary	11	1	—	—
3	2 ml	primary	12	3	—	—
4	2 ml	primary	14	4	—	—
Total: 121	2 ml	primary	6—14	121	—	—
2	12 ml	primary	8	2	—	—
1	12 ml	delayed, abortive	2 $\frac{1}{3}$	—	—	1

cates that immunity to revaccination had developed in the group.

The same holds good for the two persons, who were successfully vaccinated 2 days after inoculation of 12 ml of the gamma globulin. The third, however, who was also injected with the excessive dose of 12 ml and whose subsequent vaccination was unsuccessful, proved to be fully susceptible to revaccination 10 weeks afterwards (table 4).

It can be concluded from the data presented, that passive immunization with 2 ml of the antivaccinia gamma globulin (16 per cent) is not likely to interfere with the active immunization against vaccinia.

General reactions.

Besides the 1010 persons who were treated with various doses of gamma globulin (table 3), 537 soldiers were inoculated elsewhere with 2 ml of antivaccinia gamma globulin immediately before smallpox vaccination. In these 1551 cases no unfavourable reactions which might be due to the gamma globulin treatment were observed. The total number of persons inoculated is too small to

warrant a conclusion as to the beneficial effects of the gamma globulin. Suffice it to state that postvaccinal encephalitis has not occurred in the gamma globulin groups up to now.

DISCUSSION.

As far as can be concluded from the experience gained during the passive-active immunization trials, antivaccinia gamma globulin inoculation can be combined with smallpox vaccination without untoward reactions and without interference with the above mentioned active immunization effects. The gamma globulin, if derived from human blood, does not add a risk to smallpox vaccination. Results of a trial on a much larger scale should be awaited before its value for the prevention of encephalitis can be judged. Though there is no direct indication of specific antibodies of which a prophylactic effect might be expected, it seems reasonable, anyhow, only to use gamma globulin batches showing a high concentration of vaccinia neutralizing antibodies for trials in this field.

In the Netherlands, where the incidence of postvaccinal encephalitis among adults may be as high as 1 case in 2 to 3 thousand primary vaccinations and where this complication appeared to be independent of various modifications of home-made and foreign preparations of vaccine-lymph, a legal measure to stimulate the vaccination of infants under the age of 1 year was introduced in 1939. For those who were 2 or more years old at the time, and for those who missed being vaccinated for some reason or other, the smallpox vaccination, if required in a later period of life, involves a considerably increased risk. Prophylaxis of postvaccinal encephalitis especially in these groups is an important problem for certain countries. A passive immunization by antivaccinia gamma globulin in a dose as high as 6 ml of our preparation is worth being given a trial as a prophylactic measure. In view of the ignorance about the nature of the relation of postvaccinal encephalitis to vaccinia virus, one cannot say that smaller quantities would not have much of a chance in this respect. As 6 ml is an impracticably high dose for large scale vaccinations, an inoculation of 2 ml is recommended for future experiments.

S u m m a r y.

A batch of lyophilized vaccinia-convalescent gamma globulin (neutralization index = 10^3) was used for the passive transfer of

vaccinia neutralizing antibodies in man. Intramuscular inoculation of 2 to 3 ml of 16 per cent gamma globulin in 17 persons did not change the neutralization index of their serum. Inoculation of 6 ml, however, caused a distinct enhancement of the neutralization index in 15 out of 24 persons. Repeated determinations in two volunteers showed that the passively transferred antibodies may remain demonstrable in the blood for 2—3 weeks after the inoculation.

Vaccination with smallpox vaccine subsequent to passive immunization with gamma globulin was attempted first in a group of 1010 non-vaccinated adult emigrants. Doses of 1 to 6 ml of gamma globulin given 0 to 2 days before vaccination did not interfere with the vaccinal reaction in this group.

Active formation of vaccinia neutralizing antibodies within 2 weeks was shown in 10 out of 11 persons vaccinated one day after an inoculation of 2 ml of gamma globulin. Ten others who were treated in the same way and whose blood was examined 6 weeks afterwards, invariably showed neutralizing antibodies in their blood.

None of the 121 persons who were treated with 2 ml of the gamma globulin and vaccinated successfully 0 to 2 days afterwards, appeared to be susceptible to revaccination 6 to 14 months after the immunization. This means the development of a solid immunity to revaccination in the group.

One person, who was inoculated with a much higher quantity of gamma globulin (12 ml) 2 days before vaccination, showed a delayed abortive papular reaction, but no active formation of neutralizing antibodies after 6 weeks, nor immunity to revaccination after 10 weeks. Two others, treated in the same way with 12 ml, reacted with a typical primary response, active antibody formation and immunity to revaccination.

The conclusion may be drawn, that a moderate quantity of the antivaccinia gamma globulin, such as 2 ml (= 320 mg of dry gamma globulin), did not apparently interfere with the vaccinal reaction, nor with the development of neutralizing antibodies and immunity against revaccination in the groups of non-vaccinated adults.

Up to now a treatment with smallpox vaccine and gamma globulin has been given to 1551 persons. No untoward reactions were observed. The described technique of combined passive-active immunization can therefore be given a trial in the prevention of post-vaccinal encephalitis. For practical reasons a dose of 2 ml of anti-

vaccinia gamma globulin is recommended for future large scale experiments.

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ÉTUDE EXPÉRIMENTALE D'UNE SOUCHE BELGE d'*HISTOPLASMA CAPSULATUM*

COMPARAISON AVEC D'AUTRES SOUCHES ET AVEC *H. DUBOISII*

par

A. DUBOIS et **R. VANBREUSEGHEM**

(Reçu le 18 Avril 1955).

Nous avons eu récemment l'occasion d'isoler en Belgique une souche d'*Histoplasma capsulatum* chez un sujet porteur de lésions buccales ulcéreuses sans autres manifestations évidentes locales ou générales. Le patient était, au moment de l'apparition des lésions, rentré du Congo depuis 18 ans; nous aurions de ce fait tendance à croire qu'il s'est infecté en Belgique où il a résidé depuis lors sauf de brefs voyages en France et Hollande.

Nous avons discuté ailleurs (9) ce point qui comporte du reste 2 variantes: infection autochtone ou au contraire importée par des objets provenant de l'étranger.

Nous nous limiterons ici à la comparaison entre cette souche, des souches reçues d'Amérique tropicale de notre collègue H. FLOCH, et une souche isolée en 1952 à Anvers chez un colonial revenu récemment du Congo. Les caractères spéciaux de cette dernière souche ont amené l'un de nous à en faire une espèce nouvelle *H. duboisii* (6). Ce parasite a du reste été rencontré soit antérieurement soit postérieurement à notre isolement en diverses régions africaines et il est possible que cliniquement aussi cette infection à *H. duboisii* soit distincte de l'affection causée par *H. capsulatum* (7). Il est difficile actuellement de juger sûrement de la valeur de cette notion de „l'histoplasmose africaine" vu le petit nombre de cas connus d'infection par *H. duboisii* et d'autre part les grandes variations cliniques de l'histoplasmose classique tantôt asymptomatique, tantôt d'allure grippale tantôt réalisant la grave infection du système histiocyttaire seule décrite autrefois.

Le sujet qui nous a fourni la souche de *H. duboisii* ne semblait présenter que des lésions localisées: adénite cervicale et axillaire avec état général peu touché.

Ni *H. duboisii* ni *H. capsulatum* n'ont jusqu'à présent été isolés au Congo d'autres êtres humains ou animaux ni du sol. Un certain pourcentage d'intradermo-réactions positives à l'histoplasmine a été noté (2, 5) dans cette vaste contrée encore peu explorée à ce point de vue. Nos connaissances sur les conditions régnant en Belgique sont encore moins avancées.

MORPHOLOGIE CULTURALE DE *H. capsulatum* ET DE *H. duboisii*.

L'un de nous à l'occasion de la description d'*H. duboisii* compara la morphologie de ce champignon à celle d'*H. capsulatum*. Il nota que dans la phase mycélienne les caractères essentiels des deux espèces étaient identiques. Les deux champignons produisent en effet des microconidies et des macroconidies, ces dernières étant lisses ou échinulées. Cependant *H. duboisii* se distinguait de *H. capsulatum* par la forme des macroconidies, exclusivement rondes alors que celles de *H. capsulatum* sont rondes ou bien piriformes. Il faut bien reconnaître toutefois que cette différence morphologique était mineure et que la base essentielle de la distinction entre les deux espèces était leur morphologie à l'état parasitaire. Les souches d'histoplasmes originaires de la Guyane française et la souche isolée en Belgique avaient dans la phase mycélienne la morphologie d'*H. capsulatum* et si la présence des macroconidies piriformes est caractéristique de cette espèce elles lui appartenaient incontestablement.

On sait que sur certains milieux à la température de 37° C. *H. capsulatum* donne naissance à ce qu'on est convenu d'appeler une phase levure dont la morphologie correspond à celle du champignon à l'état parasitaire. VANBREUSEGHEM dans son travail initial sur *H. duboisii* déclare qu'il n'a pu obtenir la phase levure de cette espèce mais ultérieurement CLARKE *et al.* (3) sur une autre souche et DROUHET (com. pers.) travaillant sur la souche étudiée par VANBREUSEGHEM réussirent à obtenir la phase levure. Nous avons repris l'étude du passage de la phase mycélienne à la phase levure à propos des 4 souches de *H. capsulatum* qui font l'objet de ce travail et de la souche originale d'*H. duboisii* en utilisant le milieu proposé récemment par KURUNG et YEGIAN (11). Ce milieu fort simple résulte de l'addition à 100 ml d'une émulsion à 1% de farine de pomme de terre de 150 ml d'un mélange de 100 ml de jaune d'oeuf avec 50 ml d'oeuf entier. Ensemencées sur ce milieu à 37° C. en tubes fermés par des bouchons de liège nos quatre souches d'*H. capsula-*

tum et la souche d'*H. duboisii* se sont transformées en l'espace de quelques jours en la phase levure sans qu'il soit nécessaire pour y arriver de faire des subcultures et quel que soit l'âge de la phase mycélienne utilisée. La morphologie macroscopique des colonies ainsi obtenues est celle de colonies de levure; de couleur crème, de consistance molle et atteignant 2 à 3 mm de diamètre. Ces colonies finissent par recouvrir le milieu de culture et y forment un enduit finement mamelonné. L'examen de cette phase levure montre que les colonies de *H. capsulatum* sont constituées de cellules ayant la morphologie de l'état parasitaire: petites cellules ovoïdes de 2 à 3 μ bourgeonnant volontiers. Semblablement les colonies de *H. duboisii* examinées dans les mêmes conditions de temps sont faites de grosses cellules ovoïdes à membranes épaisses, bourrées de corpuscules de graisse et atteignant 13 à 15 μ de longueur. Cependant à côté des grandes cellules évoquant les formes parasitaires de *H. duboisii* on en trouve d'autres plus petites qui évoquent plutôt *H. capsulatum*. Les souches guyanaises et la souche belge sur milieu de KURUNG et YEGIAN ont présenté sensiblement la même morphologie, la souche belge étant peut être un peu plus grande. Dans aucune nous n'avons retrouvé la morphologie de *H. duboisii* même après plusieurs semaines. Cette opposition entre la morphologie des deux espèces à la phase levure est — pensons nous — une raison supplémentaire de croire qu'il s'agit bien d'espèces différentes.

EXPÉRIMENTATION SUR L'ANIMAL.

Nos essais ont porté sur des souris, rats, cobayes et hamsters inoculés de culture ou plus rarement de pus humain ou d'animal. Les inoculations ont été faites tant par voie péritonéale que par voie testiculaire, plus rarement par d'autres voies toujours avec la phase mycélienne.

Disons immédiatement que nous limiterons nos descriptions au cobaye et au hamster (*Mesocricetus auratus*) que nous avons surtout employés et qui nous ont donné les résultats les meilleurs: le cobaye plus spécialement pour *H. duboisii* et le hamster doré plus spécialement pour *H. capsulatum*.

INOCULATION AU HAMSTER DE 4 SOUCHES DE *H. capsulatum*.

Quatre hamsters ont été inoculés par la voie intrapéritonéale, trois avec 3 souches d'*H. capsulatum* provenant de la Guyane française, 1 avec la souche belge. A chaque hamster on a injecté un ml

de l'émulsion le 21-10-1954. Les 3 hamsters inoculés avec les souches guyanaises sont morts les 20-XI (R. 5979), 25-XI (R. 5985) et 3-XII (R. 5981). Le hamster inoculé avec la souche belge (R. 5956) est mort le 3-XII-1954. L'évolution a donc duré entre 1 mois et 6 semaines.

Un fait commun à tous les animaux inoculés était l'hypertrophie des ganglions médiastinaux. Chez les hamsters inoculés avec les souches guyanaises on a noté un foie gros et grasseux, une rate grosse, rouge et friable, quelques ganglions mésentériques. Les poumons et les reins avaient un aspect normal mais des frottis par impression ont montré la présence d'histoplasmes en abondance dans la plupart des organes. Le hamster inoculé avec la souche belge semblait normal si l'on excepte l'hypertrophie des ganglions médiastinaux dans lesquels on a trouvé de nombreux histoplasmes mais les frottis par impression des autres organes ont tous donné un résultat négatif.

EXAMEN HISTOLOGIQUE DES HAMSTERS INOCULÉS DE *H. capsulatum*.

Nous ne croyons pas devoir décrire en détail les divers animaux. Leur histoire se ressemble fort et nous nous contenterons de la description d'un d'entre eux.

Hamster doré. Inoculé de la souche 5979 par voie péritonéale le 21-X-54 et mort le 20-XI-54 soit après un mois- coupes paraffine- hématoxyline -éosine.

Foie. Il présente des lésions appréciables: nodules dans les espaces portes composés d'histiocytes et de globules blancs assez divers, même quelques polynucléaires. Seuls les histiocytes sont parasités. Les sinusoides sont dilatés, avec dans la lumière des lymphocytes et des polynucléaires du reste exempts de parasites. Les cellules de Küpfer, souvent détachées, sont volumineuses. Leur protoplasme forme une sorte de kyste. Le parenchyme hépatique est somme toute peu altéré: de l'atrophie de certaines travées et de ci de là de la métamorphose grasseuse.

Les parasites sont extrêmement nombreux: quasi toutes les cellules de Küpfer en sont chargées et souvent en nombre important (20 ou 30). Ces parasites sont très petits (entre 1 à 2 μ). Après coloration par l'hématoxyline-éosine on ne voit pas chez eux de capsules: ils montrent un pôle assez coloré et un autre non coloré. L'aspect des histoplasmes sera identique dans les autres organes.

Poumon. Les parois alvéolaires sont épaissies et riches en cellules parasitées. Il y a quelques foyers d'alvéolite exsudative mais les cellules de l'exsudat sont exemptes d'histoplasmes. Les cellules alvéolaires parasitées montrent aussi un aspect kystique mais avec des parasites moins nombreux et des dimensions plus petites qu'au foie.

Reins. Le parenchyme semble peu altéré. Les parasites sont assez nombreux, bien moins cependant que dans le foie ou la rate. On les trouve

principalement dans les cellules de l'épithélium capsulaire viscéral, plus rarement dans des cellules réticulaires isolées ou groupées entre les tubuli. On n'en voit pas dans l'épithélium capsulaire pariétal et il n'est pas établi qu'ils se voient dans les endothéliums vasculaires.

R a t e. L'hyperplasie „kystique" des cellules réticulaires donne aux coupes un aspect pâle. Entre les cellules très richement parasitées se voient encore quelques lymphocytes etc. mais les corpuscules de Malpighi ont disparu quasi totalement. L'endothélium sinusal ne semble pas parasité mais on trouve des cellules histiocytaïres parasitées libres dans les cavités sinusales.

G a n g l i o n m e d i a s t i n a l. L'aspect rappelle celui de la rate.

Deux faits frappent l'observateur: la cytologie — du reste classique — purement histiocytaire et d'autre part l'intensité du parasitisme. Il est vrai que dans le foie il y a des nodules d'allure infiltrative à cytologie complexe mais seule les histiocytes sont parasités. Les parasites, très petits ($\pm 1,6 \mu$) sont très nombreux et transforment de façon très curieuse la cellule qui prend un aspect kystique. Le noyau reste en général unique.

EXAMEN HISTOLOGIQUE DU HAMSTER INFECTÉ DE LA SOUCHE BELGE (R. 5956).

Inoculé I.P. le 21-X-54 mort le 3-XII-54. Il n'y a pas de bien grandes différences mais les parasites sont beaucoup plus rares. Le foie montre les mêmes nodules que l'animal précédent mais il faut chercher assez longtemps les parasites aussi bien dans les histiocytes de ces infiltrats que dans les cellules de Küpfer. Le nombre des parasites étant plus faible l'aspect kystique est moins apparent. Les reins et les ganglions sont assez peu parasités, le poumon et surtout la rate davantage. Les parasites sont de petite taille (vers $1 \mu 6$) sensiblement plus petits que ceux de la lésion humaine, d'évolution très chronique (3μ).

L'ensemble donne l'impression d'une moindre aptitude pathogène pour l'animal. Cependant les lésions hépatiques ne sont guère moindres que ci-dessus et la mort spontanée de l'animal n'a pas été beaucoup plus tardive.

INOCULATION AU COBAYE DE 4 SOUCHES DE *H. capsulatum*.

L'inoculation a eu lieu le 22-X-1954 en utilisant la même émulsion infectante que celle qui a été utilisée pour l'inoculation des hamsters. Chaque souche est inoculée à raison de 0.5 ml dans le testicule droit. On n'a utilisé qu'un cobaye par souche. Quinze jours après l'inoculation on ponctionne un testicule chez chaque cobaye. Les testicules sont très gros sauf celui du cobaye inoculé avec une des souches

guyanaïses (R. 5985). Cependant la ponction dans chaque cas rapportera un liquide très riche en histoplasmes qui ont l'aspect classique d'*H. capsulatum*. Chez les cobayes inoculés avec 2 souches guyanaïses (R. 5979 et R. 5985) on trouve également quelques chlamydosporos échinulées. Les mêmes ponctions seront refaites de 8 en 8 jours jusque deux mois avant l'autopsie des animaux. Chaque ponction ramènera des histoplasmes particulièrement nombreux chez le cobaye inoculé avec la souche belge (R. 5956). Les histoplasmes ont tous la morphologie classique mais ceux de la souche belge ont généralement tendance à être plus grands. Deux mois et demi après l'inoculation les histoplasmes sont devenus beaucoup plus rares.

Les 4 cobayes sont sacrifiés (chloroforme) le 4-III-55 (R. 5956), le 5-III-55 (R. 5979) et le 7-III-55 (R. 5981 et 5985). L'autopsie ne montre rien sauf chez le cobaye inoculé avec la souche belge (R. 5956) où le testicule inoculé est transformé en un sac de pus et chez un cobaye inoculé avec une souche de Cayenne (R. 5979) où le testicule renferme un petit abcès purulent: le pus de ces deux testicules est riche en histoplasmes. Les rates de ces 4 cobayes sont broyées avec de l'eau physiologique et le broyat estensemencé sur milieu de Sabouraud additionné de pénicilline (500 U par ml). Chacun de ces ensemencements a donné des colonies confluentes d'*H. capsulatum* sauf une rate (R. 5981) qui n'a donné que de rares colonies isolées. Le pus testiculaire des cobayes inoculés avec la souche belge (R. 5956) et la souche R. 5979 de Guyaneensemencé sur le même milieu a également donné des colonies d'*H. capsulatum*. Des frottis effectués avec le foie, les poumons, la rate et les reins des 4 cobayes n'ont montré après coloration par le Wright aucun parasite.

EXAMEN HISTOLOGIQUE DES COBAYES INFECTÉS DE *H. capsulatum*.

L'étude complète de ces animaux n'a pas été faite jusqu'à présent vu le caractère négatif des examens précédents. Cependant le testicule du cobaye inoculé de la souche 5979 a été examiné: l'organe est tellement caséeux qu'on ne le reconnaît plus et pas beaucoup mieux les cellules du granulome. Il paraît s'agir d'histiocytes de taille variable jusqu'à de très grandes dimensions mais formant rarement des plasmodies multinuclées. Les parasites nombreux sont intra ou extracellulaires et de dimension moyenne (environ 4μ). Malgré plusieurs mois d'évolution et une forte autolyse on ne trouve aucune

très grande forme. L'épididyme a montré quelques petits foyers inflammatoires sans parasite. Le testicule du cobaye 5956 n'a montré ni parasite ni lésion caractéristique. Enfin la rate du cobaye 5985 qui s'est montrée positive à la culture a été aussi examinée. A la loupe la coupe montrait des nodules blancs qu'on aurait pu prendre pour de petits foyers inflammatoires nécrotiques. L'examen microscopique montre qu'en réalité ce sont des corpuscules de Malpighi hyperplasiés en leur centre. L'identification de *H. capsulatum* y est restée incertaine (hématoxyline-éosine).

L'INFECTION A *H. duboisii* CHEZ LE COBAYE ET LE HAMSTER.

L'aspect histologique est ici bien différent. Nous l'avons décrit antérieurement aussi bien chez le cobaye (8, 9, 14) que chez le hamster.

Nous avons eu des résultats positifs chez le cobaye aussi bien après inoculation de culture qu'après inoculation de pus humain, chaque fois dans le testicule.

Après inoculation de culture nous avons observé d'abord un stade de réaction histiocytaire avec parasites intracellulaires assez petits, environ $3\ \mu$ que nous avons appelé forme *capsulatum*. Ultérieurement les coupes montrent un granulome constitué quasi uniquement de cellules géantes contenant de grandes formes parasitaires levuriformes, atteignant $10-15\ \mu$. Le granulome se caséfie en partie et les parasites deviennent extra-cellulaires. La dissémination parasitaire reste très faible. Nous avons cependant rencontré des lésions granulomateuses typiques dans la peau et la conjonctive. Leur mécanisme est mal précisé et peut être de cause externe.

Chez le hamster la dissémination de *H. duboisii* est un peu plus grande et se fait en particulier dans les ganglions mésentériques (après inoculation péritonéale) et plus rarement dans le foie. Ici aussi la cellule géante apparaît avec la forme *duboisii*.

Il y a dans cette évolution quelque chose de bien différent de l'infection à *H. capsulatum* du hamster, avec l'invasion massive du système réticulaire et l'allure spéciale, kystique que prennent les cellules qui restent le plus souvent uninuclées. Reprenant la terminologie que BINFORD (1) applique aux lésions humaines on pourrait dire que les hamsters infectés de *H. capsulatum* ont des lésions du type histiomycotique (avec en outre un état kystique très net). *H. duboisii* donne après un certain temps d'évolution le type granulomateux avec prédominance de la cellule de Langhans et caséification fré-

quente. C'était aussi l'aspect lésionnel rencontré chez l'homme infecté de *H. capsulatum* et présentant des manifestations très chroniques. Il semble que le type granulomateux — ici comme en d'autres infections — est l'apanage des lésions d'évolution lente. Quant au type sarcoïde aussi signalé par BINFORD et caractérisé par son infiltrat épithélioïde sans cellules de Langhans et sans caséification, nous l'avons rencontré au début de l'infection locale à *H. duboisii* (6).

Il nous est difficile de pousser plus loin l'analogie des lésions humaines et animales. Certaines des lésions décrites par BINFORD (abcès cérébraux, endocardites) ne nous sont pas connus chez l'animal non plus que les lésions vasculaires sousendothéliales rencontrées par SCHULZ (12).

DISCUSSION.

Il existe incontestablement des différences de virulence entre diverses souches d'*H. capsulatum*. Dans nos observations nous avons noté que les trois souches de Guyane se comportaient semblablement mais la souche belge s'est montrée beaucoup moins envahissante. Il faudrait cependant pour confirmer ce point utiliser un plus grand nombre d'animaux que nous ne l'avons fait. Si toutefois cette différence dans la virulence des souches que nous avons étudiées correspond à la réalité on pourrait tenter de l'expliquer par le fait que les souches guyanaises avaient été isolées du sol récemment tandis que la souche belge provient d'un malade chez qui elle semble avoir évolué d'une façon torpide depuis plusieurs années. Rappelons à ce point de vue que VANBREUSEGHEM et VAN BRUSSEL (13) ont signalé que le pouvoir pathogène des dermatophytes cultivés sur terre leur avait paru exalté par rapport à celui des dermatophytes entretenus sur les milieux usuels.

Notre intérêt se porte surtout sur les différences qui peuvent exister entre *H. duboisii* et *H. capsulatum*. Les expériences que nous avons rapportées ici montrent que *H. capsulatum* conserve à l'état parasitaire l'aspect morphologique qui lui est classiquement attribué. Jamais dans nos expériences les éléments parasitaires n'ont atteint la grandeur de ceux de *H. duboisii*. Tout au plus avons nous pu noter, particulièrement avec la souche belge tant chez l'animal que chez le malade observé, des formes parasitaires un peu plus grandes en relation peut être chez l'homme avec la prolongation de la maladie. Nous n'avons jamais eu l'occasion d'observer les

„grandes formes” signalées par CRUMRINE et KESSEL (4) et BINFORD (1) ni d'ailleurs les formes mycéliennes signalées par HUMPHREY (10). Au contraire l'inoculation de *H. duboisii* aboutit inmanquablement à des parasites ovoïdes, de 13 à 15 μ de long, entourés d'une coque épaisse et bourrés de sphérules de graisse. Ce n'est que dans les tous premiers jours qui suivent l'inoculation (vers la 1^{re} et la 2^e semaine) que l'on peut constater des formes qui ne se distinguent pas de celles de *H. capsulatum* et que l'un de nous a nommé formes capsulatum de l'*H. duboisii* pour les opposer aux formes parasitaires les plus habituelles de ce dernier qu'il a nommé formes *duboisii*. Aussi bien ces „formes capsulatum” de *H. duboisii* sont-elles aussi grandes que les plus grandes des formes classique de *H. capsulatum*. Rappelons qu'à la petite forme correspond une réaction histiocytaire et à la grande forme une réaction langhansienne. Très rapidement on voit apparaître les grandes formes et cela aussi bien dans les zones actives et bien vivantes du granulome que dans les parties caséifiées où on pourrait plus facilement imaginer les conditions des observations de BINFORD et de SCHWARZ(13).

R é s u m é.

Les auteurs ont étudié 4 souches de *H. capsulatum* dont 3 provenaient de la Guyane française et la quatrième de Belgique.

Ces 4 souches ont des caractéristiques morphologiques semblables à l'état parasitaire mais la souche belge semble moins pathogène.

Par contre ces 4 souches se distinguent nettement de notre souche de *H. duboisii*: 1) en culture au moins dans la phase levure. 2) à l'état parasitaire par la forme et la grandeur des deux champignons. 3) il semble enfin que la réaction tissulaire chez l'animal d'expérience est aussi d'un aspect différent pour chacune des deux espèces d'*histoplasma*.

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A PROPOSAL FOR AMENDMENT OF THE DIAGNOSIS OF THE GENUS *PICHIA* HANSEN

by

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The yeast genus *Pichia* was created by HANSEN in 1904. A detailed historical survey of the genus has recently been given by LODDER and KREGER-VAN RIJ (3), who now define it as follows :

“Cells of various shape, oval to long-cylindrical. Vegetative reproduction by multilateral budding. Pseudomycelium formation.

Heterogamous conjugation may or may not immediately precede ascus formation. The spores are hemispherical, hat-shaped, angular or round, usually with an oil drop inside. One to four spores in the ascus.

In liquid media a dry dull pellicle is formed.

Metabolism preferentially oxidative. However, fermentation may also occur¹). Nitrate not assimilated. Splitting of arbutin generally absent or only weak.”

Recently a number of new species of yeast have been described which appear to be closely related to members of the genus *Pichia* as defined above. These species are characterized by the formation of hat- or helmet-shaped ascospores, absence of fermentation and inability to utilize nitrate. Yet these organisms defy classification in *Pichia* because a dry, dull pellicle on malt extract is not formed, the pseudomycelium is very reduced or absent or both properties may be lacking. SHIFRINE and PHAFF (6) described *Pichia haplophila*, a species isolated from bark beetles, which lacks a pseudomycelium but does form a pellicle on malt extract. PHAFF and KNAPP (4) discovered two additional species in slime fluxes of the black oak,

¹) This is especially true of aerobically grown cells which under suitable conditions may give rise to a slow and only gradually increasing fermentation.

Quercus kelloggii. These two new species, described as *Pichia quercibus* and *Pichia carsonii*, form very primitive pseudomycelia and do not produce a pellicle in liquid wort. PHAFF, MILLER and SHIFRINE (5) isolated and described *Pichia xylosa*, a new species of *Pichia* which forms a well developed pseudomycelium, but also fails to form a pellicle on malt extract medium.

It seems inadvisable to create a new genus to accomodate these species. The differences in pellicle formation are not sharp but rather overlapping in the different species from heavy to thin or none at all. A similar range of differences exists with respect to pseudomycelia, which may be well developed, primitive or completely absent. It is therefore proposed to amend the definition of the genus *Pichia*.

The diagnosis of the amended genus *Pichia* is as follows: Cells vary from short oval to long cylindrical. Vegetative reproduction by multilateral budding. Usually pseudomycelium formation, although the pseudomycelium may be very primitive or even absent.

Heterogamic conjugation may or may not immediately precede ascus formation. The spores are helmet-shaped, hat-shaped or spherical. Usually four spores per ascus. In liquid media a dry dull pellicle is often formed, but the pellicle may be thin or even lacking. Metabolism preferentially oxidative, although fermentation occurs in some species.

Nitrate not assimilated. Splitting of arbutin generally absent or only weak.

If the new definition of *Pichia* is followed, it becomes possible to transfer *Saccharomyces pastori* and *Saccharomyces pini* to the genus *Pichia*. Both species, which have their natural habitat in the exudates or phloem sap of trees, produce typical hat-shaped spores which are readily liberated from the ascus. *S. pastori* ferments glucose only and *S. pini* ferments glucose very weakly or not at all. Because of these two properties they are very atypical members of the genus *Saccharomyces*; in fact, they are the only members of this genus with hat-shaped spores. LODDER and KREGER - VAN RIJ considered *S. pastori* and *S. pini* to be identical (3), but WICKERHAM (8) has presented evidence that they are distinct species. We concur with WICKERHAM since the isolates of *S. pini* reported by SHIFRINE and PHAFF (6) and the isolates of *S. pastori* obtained by PHAFF and KNAPP (4), corresponded exactly with the complete description of these two yeasts kindly supplied by Dr WICKERHAM. The ability

of *S. pastori* to ferment glucose is no objection to its transfer to *Pichia*, since the latter genus already contains one species which can ferment glucose rapidly (*P. fermentans*). In view of these considerations we feel that *S. pastori* and *S. pini* are more closely related to *Fichia* than to *Saccharomyces* and these organisms will therefore be designated as *Pichia pastori* (Guilliermond) nov. comb. and *Pichia pini* (Holst) nov. comb. Neither *P. pastori* nor *P. pini* forms a pellicle or a pseudomycelium.

Pichia pastori appears to have its natural habitat in the exudate of certain deciduous trees. The first isolation of this yeast was made by GUILLIERMOND (1), who isolated it from the exudate from a *Aesculus* tree. The California isolates were all obtained from the black oak. *Pichia pini*, on the other hand, has been isolated only from coniferous trees (2,6).

The author would like to suggest transfer of one other yeast, *Debaryomyces vini*, to the genus *Fichia*. This yeast, listed by LODDER and KREGER - VAN RIJ (3, pg. 292) under *Debaryomyces*, forms a well-developed pseudomycelium and produces 4 spores per ascus. Both properties are atypical in the genus *Debaryomyces* and as LODDER and KREGER - VAN RIJ have pointed out, *D. vini* takes a place intermediate between the genera *Debaryomyces* and *Pichia*. The only reason why these authors did not include *D. vini* in *Pichia*, was the fact that *D. vini* fully lacked the property to form a pellicle on malt extract, making the inclusion in *Pichia* impossible. However, if the amended diagnosis of *Pichia* is adopted, there are no more objections to such a transfer. In line with this, it is proposed to transfer *D. vini* to *Pichia* as *Pichia vini* (Zimmermann) nov. comb.

A similar modification of the diagnosis of the genus *Hansenula* was made by WICKERHAM (7) who discovered a number of new species of that genus which lacked the ability to form pseudomycelia or pellicles. Such species had not been known until that time and necessitated amendment of the genus *Hansenula*. It seemed logical, therefore, to broaden the definition of *Pichia* on similar grounds. It may become possible to postulate an evolutionary line of development for *Pichia* as was done for *Hansenula* by WICKERHAM (7), when a sufficient number of accurately described species becomes available.

The principal difference remaining between *Pichia* and *Debaryomyces* following the amended diagnosis for *Pichia*, is the formation of the characteristic asci in *Debaryomyces* in which a single (or

rarely two), occasionally warty ascospore is produced after conjugation involving usually a mother and daughter cell.

S u m m a r y.

An amended diagnosis has been given for the genus *Pichia* Hansen to allow inclusion of species characterized by a lack of pellicle formation, by the absence or a strongly reduced pseudomycelium or by a combination of both properties. The following species fit the amended diagnosis of *Pichia*: *P. haplophila* Shifrine et Phaff, *P. carsonii* Phaff et Knapp, *P. quercibus* Phaff et Knapp and *P. xylosa* Phaff, Miller et Shifrine. Furthermore, it has been proposed to transfer *Saccharomyces pastori* Guilliermond, *Saccharomyces pini* Holst and *Debaryomyces vini* Zimmermann to the amended genus *Pichia*, where they fit well rather than occupying an exceptional position in their present genera.

A d d e n d u m.

In a recent paper by J. BOLDIN and F. ABADIE (Bull. Soc. Mycol. France **70**, 353, 1954) a new genus, *Petasospora*; has been proposed to accomodate those species of *Saccharomyces* which have hat-shaped spores and which lack fermentative power or ferment weakly. Several new species isolated from tanning liquors were also placed in this genus. It would seem to the author that there is no real need to establish a separate genus for this group of yeasts which could be accomodated in the amended genus *Pichia* as suggested in this article. Finally, *Debaryomyces marama* described by M. E. DI MENNA (J. Gen. Microbiol. **10**, 65, 1954) can also be transferred to *Pichia*, since it seems to be more similar to the spherical spored species of *Pichia*, than to typical forms of *Debaryomyces*.

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THE TAXONOMY OF YEASTS FOUND IN EXUDATES OF CERTAIN TREES AND OTHER NATURAL BREEDING SITES OF SOME SPECIES OF *DROSOPHILA*

by

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INTRODUCTION.

In a separate publication, CARSON, KNAPP and PHAFF (1) described the yeast flora of the natural breeding sites of some species of *Drosophila* in the Yosemite region of California. It was found that the yeasts isolated constituted a typical and specific flora, differing considerably from the yeast flora found in the crops of adult specimens of *Drosophila* occurring in the same region (7). One hundred and ten of the 134 cultures were isolated from slime fluxes of *Quercus kellogii* (the black oak) and *Abies concolor* and the remainder from a rotting log of *Populus tremuloides* and from certain *Clavaria* and *Pleurotus* fungi. Emphasis in the previous paper (1) was on the relation of the larval stages of *Drosophila* to the yeasts, which form an important part of their natural food supply. The present publication will deal with the taxonomy of the yeasts and a description is given of four new species isolated from slime fluxes.

METHODS.

Details of the isolation procedure have been described previously (1). The identifications were done by the system of LODDER and KREGER - VAN RIJ (4) and by WICKERHAM's (12) procedure for *Hansenula*. In addition, the assimilation patterns of yeasts not belonging to *Hansenula* were also studied using the much larger

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number of carbon compounds advocated by WICKERHAM for a more clear cut differentiation of species. All the carbon compounds used for assimilatory studies are listed in Table 2. In the description of new species negative assimilation results are not recorded. Variable assimilation means that in a number of strains of the same species a particular compound may be utilized by some strains and weakly or not at all by others. The initial assimilation tests were done by the replica plating procedure of SHIFRINE, PHAFF and DEMAINE (10). To eliminate possible erroneous readings due to interference between colonies on certain sugars, confirmatory tests were made for all new species in liquid synthetic media in test tubes (12). Sugars most apt to cause interference between colonies on plates (10) are sucrose and raffinose; more rarely interference was observed with other di- or trisaccharides. Such interference is due to a more rapid splitting of a disaccharide than utilization of the resulting hexoses by the cells. In such cases, diffusion of the liberated hexoses to neighbouring colonies can lead to false positive reactions. Other additional tests applied to all new species are their ability to grow in a vitamin free medium and the production of acid on chalk agar. This medium (5 % glucose, 0.5 % Difco yeast extract, 0.5 percent precipitated calcium carbonate) was poured in petri dishes and usually six pinpoint inoculations were made on each surface. A 7 to 10 mm clearing around a colony in one week is interpreted as strong acid production. The Dalmau coverslip technique (12) for anaerobic pseudomycelium formation was applied to the streaks on potato-dextrose agar.

EXPERIMENTAL RESULTS.

The yeasts isolated, the number of strains of each species and the sources of isolation are recorded in Table 1.

In the genus *Saccharomyces* only two isolates were obtained, each representing a separate species. *Saccharomyces elegans* was described in 1952 by LODDER and KREGER - VAN RIJ (4) and was originally isolated from apple juice in Holland. Our culture fits their description very well, including its abundant sporulation on malt agar even after three years from the date of isolation. In order to make the identity of the two strains more certain, an authentic culture of the Dutch strain was obtained and their carbon assimilation patterns were compared. These proved to be identical in most respects. Since this information is not available in the literature,

TABLE 1.
Yeasts isolated and sources of isolation in the Yosemite Region, Sierra Nevada Mountains, California.

Name	No. of cultures identified	Sources of isolation ¹⁾
<i>Saccharomyces elegans</i> Lodder et Kreger - van Rij 1952	1	Slime flux of <i>Quercus kelloggii</i>
<i>Saccharomyces drosophilae</i> Shehata et al. 1955	1	Slime flux of <i>Quercus kelloggii</i>
<i>Hansenula mrakii</i> Wickerham 1951 (imperfect form)	31	Slime fluxes of <i>Quercus kelloggii</i>
<i>Hansenula minuta</i> Wickerham 1951 (imperfect form)	8	Decaying bark from partially submerged, fallen, dying <i>Populus tremuloides</i> , Yosemite Creek
<i>Debaryomyces fluxorum</i> nov. spec.	35	Slime fluxes of <i>Q. kelloggii</i> and <i>Abies concolor</i>
<i>Pichia silvestris</i> nov. spec.	17	Slime fluxes of <i>Q. kelloggii</i> and <i>Abies concolor</i>
<i>Pichia carsonii</i> nov. spec.	3	Slime fluxes of <i>Q. kelloggii</i>
<i>Pichia quercibus</i> nov. spec.	1	Slime flux of <i>Q. kelloggii</i>
<i>Pichia polymorpha</i> Kloecker 1912	2	Rotten logs of <i>Pinus contorta</i> and <i>Abies</i> species.
<i>Pichia pastori</i> (Guilliermond) nov. comb. 1919	11	Slime fluxes of <i>Q. kelloggii</i>
<i>Endomyces javanensis</i> Kloecker 1909	3	Slime fluxes of <i>Q. kelloggii</i>
<i>Candida humicola</i> (Daszewska) Diddens et Lodder 1912	5	From species of <i>Pleurotus</i> and <i>Clavaria</i> fungi
<i>Candida mycoderma</i> (Rees) Lodder et Kreger-van Rij 1870	5	Slime fluxes of <i>Q. kelloggii</i>
<i>Candida parapsilosis</i> (Ashf.) Lang. et Talice var. <i>intermedia</i> van Rij et Verona 1949	1	Slime fluxes of <i>Q. kelloggii</i>
<i>Trichosporon fermentans</i> Diddens et Lodder 1942	7	Slime fluxes of <i>Q. kelloggii</i>
<i>Torulopsis inconspicua</i> Lodder et Kreger - van Rij 1952	1	Slime fluxes of <i>Q. kelloggii</i>
<i>Cryptococcus laurentii</i> (Kuff.) Skinner var. <i>magnus</i> Lodder et Kreger-van Rij 1952	1	Slime fluxes of <i>Q. kelloggii</i> Tunnel of bark beetles in <i>Abies magnifica</i>
<i>Sporobolomyces roseus</i> Kluyver et van Niel 1924-1925	1	
Unidentified	2	
Total	136	

¹⁾ For a more detailed description of the areas and sources of isolation see reference (1).

Table 2 gives the assimilation pattern of this yeast and of several other species isolated. A single isolate was obtained of *Saccharomyces drosophilarum*, a yeast with kidney-shaped ascospores. Our culture checked in all respects with the standard description of this new yeast by SHEHATA *et al.* (8).

Genus Endomyces. Three cultures were obtained which are very similar to *Endomyces javanensis* (syn. *Endomycopsis javanensis*). All three cultures were isolated from a slime flux on the same oak, but at different times. Two isolates were obtained during the early and middle part of the summer of 1951. The third isolate was obtained in 1952. Although the same tree was sampled extensively in 1954 and the original flux was quite active, this species was not isolated again. We prefer to call these isolates *Endomyces javanensis* as KLOECKER originally named it, rather than *Endomycopsis javanensis* as was done by STELLING-DEKKER (11) and LODDER and KREGER-VAN RIJ (4). According to the definition of *Endomycopsis*, budding cells should be formed.

However, budding was not apparent in our isolates or in an authentic culture obtained from the C.B.S.¹⁾ collection or in the illustrations of this yeast by LODDER and KREGER-VAN RIJ. In fact, LODDER and KREGER-VAN RIJ specifically mentioned (4, pg. 107) that budding cells were absent in slide cultures. Certain hyphal swellings (usually terminal) are sometimes formed, which resemble buds to some extent, but these usually elongate and are cut off by the formation of a septum rather than being pinched off as is typical in the case of true budding. In our strains, sporulation occurred most commonly without any evidence of conjugation (in a hyphal swelling), but also after a type of conjugation in which the zygote looks like the letter H. One of the conjugating cells then swells slightly and four oval, slightly flattened spores are formed. These conjugating structures might well be compared to primitive gametangia, one of the characteristics of *Endomyces*. Saturn-shaped spores or a warty spore surface, which were observed by LODDER and KREGER-VAN RIJ (4, pg. 108), have not been observed in our strains. The asci rupture readily at maturity. The carbon assimilation patterns of the KLOECKER strain and the California strains were found to be almost identical (see Table 2).

Genus Pichia. Five species were isolated which we con-

¹⁾ Centraal Bureau voor Schimmelcultures, Baarn, Holland.

sider members of the genus *Pichia*. The two isolates of *P. polymorpha* agree well with the description given by LODDER and KREGER - VAN RIJ (4), although complete carbon assimilation reactions were not compared. Three species could not be identified with any of the known forms of *Pichia* and will therefore be described as new species.

Pichia silvestris nov. spec. (represented by 17 isolates) forms helmet- to hat-shaped ascospores, does not ferment, produces a well developed pseudomycelium and a thin film on malt extract. It is therefore a typical representative of the genus. The cultures resemble *P. membranaefaciens* except that the cells are smaller in malt extract and long-oval or cylindrical cells, which are typical for *P. membranaefaciens*, are lacking in the new species. Furthermore, none of the isolates of *P. silvestris* can utilize glycerol, whereas this compound can serve as a single carbon source for *P. membranaefaciens* according to WILES (15).

Standard description of *Pichia silvestris* nov. spec.

Growth in malt extract: After 3 days at 25°C. cells are globose to oval $(1.8 - 5.3) \times (3.3 - 7.0) \mu$. Rarely the length of a cell may go up to 9.0μ . The usual ratio between length and width is 1.5. The thallus consists of irregularly branched clusters or cells occur singly and in pairs. A thin, dull smooth film is formed. After 3 weeks, a ring, sediment and a very thin film are present.

Growth on malt agar: Buff to brownish; center smooth, semi-glossy; periphery reticulate, dull with semi-glossy smooth sectors; butyrous texture, convex cross section, rather spreading; margin lobulate with a short fringe of pseudomycelium.

Slide cultures on potato dextrose agar: Pseudomycelium well developed, of the *Candida*-type. Not much differentiation between blastospores and pseudomycelial cells. Many of the latter contain ascospores.

Sporulation: Abundant on malt agar, helmet- to hat-shaped, 2 to 4 (mostly 4) per ascus. Spores formed without previous conjugation of vegetative cells, but also after heterogamic conjugation. Asci rupture readily at maturity.

Fermentation: Absent.

Assimilation of carbon compounds: Only glucose, ethanol (with formation of a pellicle), lactic acid and succinic acid are utilized. No growth occurred with any of the other carbon sources tested. Splitting of arbutin negative.

Utilization of KNO_3 : Absent.

Growth in a vitamin free medium: Positive.

Acid production on chalk agar: Negative.

Natural habitat: Slime fluxes of *Quercus kelloggii* and *Abies concolor*. Culture K-358 has been chosen as the type strain of this species.

Pichia quercibus nov. spec., represented by a single isolate, forms hat-shaped spores, does not ferment, does not utilize nitrate, forms a very primitive pseudomycelium, but no pellicle is produced on malt extract. Species of *Pichia* devoid of pseudomycelium and film forming ability cannot be included in the genus *Pichia* as defined by LODDER and KREGER-VAN RIJ (4). However, they may be accommodated in the amended diagnosis of this genus as proposed by PHAFF (6).

Standard description of *Pichia quercibus* nov. spec.

Growth in malt extract: After 3 days at 25°C. cells small, globose, spherical or short oval, $(1.8 - 5.6) \times (1.8 - 6.1) \mu$ singly or pairs or in small clusters, slight ring, no pellicle. After 3 weeks, a well developed ring and sediment are present, but no pellicle (rarely islets).

Growth on malt agar: After 1 month at 25°C. the streak culture is buff colored, surface semi-glossy and nearly smooth, texture butyrous, cross section broadly convex, rather spreading, margin slightly irregular but no pseudomycelium present.

Slide cultures on potato dextrose agar: A very primitive pseudomycelium is formed aerobically, showing little differentiation between blastospores and pseudomycelial cells.

Sporulation: Abundant on malt agar in 3 days. Spores hat-shaped, usually 4 per ascus. Iso- or heterogamic conjugation usually precedes ascus formation, although some asci form without immediately preceding conjugation. The asci rupture readily at maturity.

Fermentation: Absent or a very weak fermentation of glucose after several weeks.

Assimilation of carbon compounds: Glucose, cellobiose, ethanol, glycerol, D-mannitol, D-sorbitol, salicin (latent), gluconic acid (weak), K-5-keto-gluconate, lactic and succinic acids are assimilated. Pellicles are not produced on the assimilation media. The other compounds tested were not utilized. Splitting of arbutin positive.

Growth in a vitamin free medium: Positive.

Assimilation of KNO_3 : Negative.

Acid production on chalk agar: Moderate.

Isolated from slime flux of the black oak *Quercus kelloggii*.

Pichia carsonii nov. spec., represented by three isolates, forms helmet-shaped spores (only after iso- or heterogamic conjugation), does not ferment and forms a very primitive pseudomycelium. This species, like *P. quercibus*, does not form a film on liquid wort. It

differs from the latter primarily in its ability to assimilate a rather large number of carbon compounds.

Standard description of *Pichia carsonii* nov. spec.

Growth in malt extract: After 3 days at 25°C. cells small, globose to short oval, $(1.8 - 5.6) \times (2.1 - 6.2) \mu$, singly, in pairs or short chains. A poorly developed irregular ring is present but no pellicle. After 3 weeks a fairly well developed ring, a sediment but no pellicle.

Growth on malt agar: Light cream-colored; surface smooth, semi-glossy, with a few sectors in the margin. Texture soft. Cross section slightly raised to flat, little spreading. Margin lobate with a short pseudomycelial fringe.

Slide culture on potato dextrose agar: Pseudomycelium, which is of the *Candida*-type, is very primitive. Blastospores and pseudomycelial cells are almost identical in appearance.

Sporulation: Rather abundant on malt agar in 3 days. In each ascus, usually four helmet-shaped ascospores with a short brim and a small refractive granule. Iso- or heterogamic (mother-daughter cell) conjugation precedes sporulation. The asci rupture readily at maturity.

Fermentation: Absent.

Assimilation of carbon compounds: Glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, melezitose, soluble starch, D-xylose, L-arabinose, D-ribose (variable), ethanol (a shiny, fragile pellicle is formed, which easily sinks to the bottom), glycerol (weak), adonitol, D-mannitol, D-sorbitol, alpha-methyl-D-glucoside, salicin, gluconic acid (latent), Ca-2-keto-gluconate, DL-lactic acid (weak), succinic acid and citric acid. Splitting of arbutin positive. The other compounds are not utilized.

Growth in a vitamin free medium: Positive.

Assimilation of KNO_3 : Absent.

Acid production on chalk agar: Negative.

Isolated from a slime flux of the black oak *Quercus kelloggii*.

It is named in honor of Professor H. L. CARSON in recognition of his work, on the ecology of *Drosophila*. Culture K-31 has been chosen as the type strain of this species.

Eleven cultures were isolated from *Quercus kelloggii* which corresponded to *Saccharomyces pastori* Guilliermond. However, in accordance with the proposed amendment of the genus *Pichia* (6), these isolates will be transferred to the genus *Pichia* and are designated as *Pichia pastori* (Guilliermond) nov. comb. Since the complete carbon assimilation pattern of this yeast has not been reported previously it is listed in Table 2. LODDER and KREGER - VAN RIJ

regarded *S. pastori* and *S. pini* (3) as identical species, but WICKERHAM (13) has presented evidence that they are distinct species. We concur with WICKERHAM since the properties of the isolates of *S. pini* reported by SHIFRINE and PHAFF (9) and those of *S. pastori* obtained in the present survey, corresponded exactly with the complete descriptions of these two yeasts kindly supplied by Dr WICKERHAM (see Table 2 for a comparison of the two species). In accordance with the amended diagnosis of *Pichia* (6), *Saccharomyces pini* also will be transferred to *Pichia*.

TABLE 2.

Assimilation of carbon compounds by various yeasts. V = variable and indicates that the compound is used by some strains and not or weakly by others. L = latent growth. W = weak growth

	D-glucose	D-galactose	L-sorbose	Maltose	Sucrose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-xylose	L-Arabinose	D-Arabinose	D-Ribose
<i>S. elegans</i>	+	L	V	—	W	—	L	—	—	—	—	—	—	—	—	—	—
<i>P. pastori</i>	+	—	—	—	—	—	L or +	—	—	—	—	—	—	—	—	—	—
<i>P. pini</i>	+	—	—	—	—	+	+	—	—	—	—	—	—	L	V	—	+
<i>E. javanensis</i>	+	W	+	—	—	—	+	—	—	—	—	—	—	W	—	—	—
	L-Rhamnose	Ethanol	Glycerol	D-Erythritol	Adonitol	Dulcitol	D-mannitol	D-sorbito	α -methyl-D-glucoside	Salicin	Gluconic acid	Ca-2-keto-glucuronate	K-5-keto-glucuronate	DL-lactic acid	Succinic acid	Citric acid	D-Inositol
<i>S. elegans</i>	—	—	W	—	+	—	+	+	—	—	+	W	—	—	—	—	—
<i>P. pastori</i>	+	+	+	—	—	—	+	L or +	—	—	V	—	—	L	+	—	—
<i>P. pini</i>	+	+	L	+	+	—	+	+	—	+	—	V	—	—	V	V	—
<i>E. javanensis</i>	—	W	+	—	—	—	—	—	—	—	—	—	—	+	+	—	—

Genus *Debaryomyces*. Thirty-five isolates were obtained from slime fluxes of *Quercus* and *Abies* which fit well in the genus *Debaryomyces* as defined by LODDER and KREGER - VAN RIJ. The cultures are all similar except for variable utilization of adonitol and lactic acid, which we do not consider sufficient for separation into two species. Since only glucose is utilized among the sugars used by the Dutch workers, the present isolates cannot be identified with

any of the described species. We propose the name *Debaryomyces fluxorum* because of their frequent occurrence in tree exudates.

Standard description of *Debaryomyces fluxorum* nov. spec.

Growth in malt extract: After 3 days at 25°C. cells are usually oval $(2.1 - 5.3) \times (3.5 - 8.9) \mu$, mostly occurring singly, in pairs or in chains of three. A thin, dull, smooth, creeping pellicle is formed.

Growth on malt agar: After 3 weeks at 25°C., the color is buff, surface smooth and semi-dull. Texture butyrous. Cross section nearly flat to broadly convex. Margin entire to somewhat irregular.

Slide cultures on potato dextrose agar: A very primitive pseudomycelium may be formed irregularly along the streak. Slightly better development under anaerobic conditions (under coverslip).

Sporulation: Occurs on vegetable agar and malt agar, but is not abundant. Usually two, sometimes one spherical spore per ascus. The spore wall is more or less rough and there is a distinct refractive granule in the center of each spore. Conjugation prior to sporulation has not been observed. Rupturing of the asci does not occur at maturity.

Fermentation: Absent.

Assimilation of carbon compounds: Of the 34 compounds tested, assimilation is positive with glucose, ethanol (with the formation of a thin, dull, corrugated film), adonitol (variable), D-mannitol, D-sorbitol, DL-lactic acid (weak or negative) and succinic acid. Splitting of arbutin negative.

Assimilation of KNO_3 : Negative.

Production of acid on chalk agar: Very weak.

Growth in vitamin free medium: Negative.

The natural habitat of this yeast appears to be in slime fluxes of *Quercus kelloggii* and *Abies concolor*.

Culture K-369 (which is adonitol positive) has been chosen as the type strain of this species.

Genus *Hansenula*. Although no cultures were isolated which belong to *Hansenula* in the strict sense, a number of nitrate positive isolates were obtained which were unable to sporulate under our conditions but appeared to be identical in most other respects with described species of *Hansenula*. The two species which the new isolates resemble are *Hansenula mrakii* (31 isolates) and *Hansenula minuta* (8 isolates). Our cultures fit the descriptions given by WICKERHAM (12) of these two species, including their carbon assimilation patterns, except that our isolates require an exogenous supply of vitamins. This indicates a more primitive character ac-

cording to WICKERHAM. We considered the possibility that our isolates might be heterothallic haploids with regard to sex and have therefore mixed the isolates of each series in groups of four and eight (14). However, no sporulation was observed. Since WICKERHAM (personal communication) has similar isolates in his collection, representative cultures from different trees have been sent to his laboratory in the hope that mating types might be found. Since there is such close similarity between our cultures and the perfect species in *Hansenula*, we prefer at this time not to describe these yeasts as new imperfect species.

The remaining species listed in Table 1, *Candida humicola*, *Candida mycoderma*, *Candida parapsilosis* var. *intermedia*, *Trichosporon fermentans*, *Torulopsis inconspicua*, *Cryptococcus laurentii* var. *magnus* and *Sporobolomyces roseus*, all corresponded quite well with the descriptions given by LODDER and KREGER - VAN RIJ (4).

LATIN DIAGNOSES OF NEW SPECIES.

Pichia silvestris nov. spec.

In musto maltato cellulæ ovoideae, $(1.8 - 5.3) \times (3.3 - 7.0) \mu$, nonnulae 9μ longae, singulae, binae aut catenatae. Post dies 3-4 pellicula non-nitida, tenuis, non-crispulata formatur. Sedimentum. Cultura in agaro maltato (post unum mensem) flavifusca, glabra, mollis, parum nitida, margine piloso. Pseudomycelium abundat. Cellulae pseudomycelii et blastosporae longovoideae aut oblongae in catenis ramosis. Ascosporae pileiformae ad 4 in quoque asco. Plerumque formantur asci ex transformatione cellularum vegetativarum diploidearum. Pauci asci ex conjugatione cellularum haploidearum. Fermentatio nulla. In medio minerali cum glucoso, alcohole aethylico (pellicula formatur), acidum lacticum, acidum succinicum crescit. Arbutinum non finditur. Nitras kalicus non assimilatur. Isolata ex *Quercus kelloggii* at *Abies concolor* in Mather, California.

Pichia quercibus nov. spec.

In musto maltato cellulæ subovoideae aut rotundae $(1.8 - 5.6) \times (1.8 - 6.1) \mu$, singulae, binae aut catenatae. Sedimentum anulusque formantur. In agaro maltato cultura (post unum mensem) flavifusca, mollis, prope glabra, parum nitens, margine parum undulato. Pseudomycelium nullum; interdum cellulæ in catenis parvis. Plerumque formantur asci ex conjugatione cellularum haploidearum, at pauci asci ex transformatione cellularum vegetativarum diploidearum. Pileiformae ascosporae ad 4 in quoque asco. Fermentatio nulla aut exigua glucosi solius. In medio minerali glucosum, cellobiosum, alcohole aethylicum, glycerolum, mannitolum, sorbitolum, salicinum (exiguum), acidum gluconicum (exiguum), K-5-ketogluconicum, acidum lacticum, acidum succinicum assimilantur at non nitras kalicus. Pelliculae non formatur. Isolata ex *Quercus kelloggii* in Mather, California.

Pichia carsonii nov. spec.

In musto maltato cellulae subovoideae ($1.8 - 5.6$) \times ($2.1 - 6.2$) μ singulae, binae aut catenatae brevae. Sedimentum anulusque formantur. Pellicula deficiunt. In agar o maltato (post unum mensem) flavalbida, glabra, prope nitida, mollis, prope plana, margine undulato aut pseudomycelium primitivum. Pseudomycelium primitivum cellulis aequis ovoidisque. Conjugatio heterogamica inter cellulas maternas et earum gemmas aut conjugatio isogamica. Pileiformae ascosporae ad 4 in quoque ascus. Fermentatio nulla. In medio minerali glucosum, galactosum, sorbosum, maltosum, saccharosum, cellobiosum, trehalosum, melezitolum, amyllum, xylosum, L-arabinosum, D-ribosum (var.), alcohole aethylicum (pellicula), glycerolum (exigua), adonitolum, mannitolum, sorbitolum, alpha-methylglucosum, salicinum, acidum gluconicum (exigua), Ca-2-ketogluconicum, acidum lacticum (exigua), acidum succinicum, acidum citricum assimilatur at non nitras kalicus. Isolata ex *Quercus kelloggii* in Mather, California.

Debaryomyces fluxorum nov. spec.

In musto maltato cellulae ovoidae ($2.1 - 5.3$) \times ($3.5 - 8.9$) μ , plerumque singulae aut binae. Pellicula tenuis, non-nitida, glabra, sursum repens formatur. Sedimentum. Cultura in agar o maltato (post unum mensem) flavifusca, glabra, parum nitida, mollis, margine parum undulato. Pseudomycelium primitivum; cellulae in catenis parvis. Asci formantur ex transformatione cellularum vegetativarum diploidearum. Ascosporae rotundae, 1-2 in quoque asco. Fermentatio nulla. In medio minerali glucosum, alcohole aethylicum (pellicula tenuis, non-nitida, crispulata), adonitolum (var.), mannitolum, sorbitolum, acidum lacticum (exiguum aut nullum), acidum succinicum assimilatur at non nitras kalicus. Arbutinum non finditur. Isolata ex *Quercus kelloggii* et *Abies concolor* in Yosemite, California.

DISCUSSION.

Identification of the yeasts occurring in slime fluxes of the black oak, *Quercus kelloggii*, has shown that a number of species of yeast could not be placed in any of the existing genera of the *Saccharomycetaceae*. By modifying the definition of the genus *Pichia* (6), to allow the inclusion of species which do not form a pseudomycelium or a pellicle on malt extract, it became possible to place *Pichia quercibus* and *P. carsonii* in this genus and to transfer *Saccharomyces pastori* from *Saccharomyces* to the genus *Pichia*. *P. pastori* appears to have its natural habitat in the exudates of deciduous trees. The first isolation was made by GUILLIERMOND from the exudate of a *Aesculus* and the California isolates were all obtained from the black oak.

The most common genera found in slime fluxes are *Pichia*, *Debaryomyces* (represented by a single new species) and yeasts which

are presumably imperfect representatives of *Hansenula mrakii*. The last species was originally isolated from soil in New Guinea, where it may have been introduced by an exuding tree, just like one of our isolates of *H. mrakii* was obtained from moist soil underneath a dripping slime flux. It seems very likely that *Candida mycoderma* and *Torulopsis inconspicua* represent imperfect forms of the genus *Pichia* as defined by us. Thus, the yeast flora comprising certain species in the genera *Pichia*, *Debaryomyces* and *Hansenula* appears to be quite specific in fluxes of *Q. kelloggii* and *A. concolor* in the area studied. This is further supported by the fact that all common species were isolated in two different years (1952 and 1954). The remainder of the isolates not included in the above genera represent small numbers of a variety of species.

LUND (5) recently reviewed the general ecology of yeasts and he also studied briefly the yeasts in exudates of various deciduous trees. Although the literature (*cf.* LODDER and KREGER-VAN RIJ, 4) indicates that the natural habitat of the genera *Saccharomyces* and *Nadsonia* is in the exudates of deciduous trees, including *Quercus*, no species of these two genera were found by LUND or by us. It is very possible that the "Mycodermas" and species of *Torulopsis* mentioned in the older literature may have been imperfect forms of *Pichia*. As early as 1899 HANSEN (2) described the occurrence of *Pichia membranaefaciens* and similar imperfect forms in slime fluxes. LUND (5) found only *Candida mycoderma* in *Quercus*. Our survey yielded five isolates of this yeast. Of the five isolates of *C. humicola*, three were isolated from *Pleurotus* fungi and two from *Clavaria* fungi. It is interesting to note that one of the C.B.S. strains (4, pg. 528) of this yeast was isolated also from mushrooms. *Debaryomyces fluxorum* is the first species of this genus isolated from a slime flux. In general, the yeast flora is composed of non-fermentative or poorly fermentative species. This is in striking contrast with the rather strongly fermentative yeast flora isolated from the crops of *Drosophila* collected in the same region (see the accompanying publication by PHAFF, MILLER and SHIFRINE). The non-identity of the two floras has been taken as evidence that the species of *Drosophila* studied do not feed on the yeasts in slime fluxes although some species use the fluxes for oviposition and larval development (1).

It is interesting and significant that the application of a large number of carbon compounds in the assimilation tests has not lead

to an undesirable and impracticable splitting into dwarf species as was feared by LODDER and KREGER - VAN RIJ (4, pg. 3). The best proof that the system has definite merits is the finding of groups of as many as 30 and 35 isolates with only minor variations in their carbon assimilation patterns.

S u m m a r y.

A survey was made of the yeasts occurring in slime fluxes of *Quercus kelloggii* (black oak), *Abies concolor* (red fir) and in certain mushrooms and dead logs in the mountains of the Yosemite region of California. Most of the 134 isolates identified were found to be non-fermentative or poorly fermentative (fermenting glucose only and usually weakly). The isolates were placed in the following genera: *Pichia* (34), *Debaryomyces* (35), imperfect forms of *Hansenula* (39), *Endomyces* (3), *Saccharomyces* (2), *Candida* (11), *Trichosporon* (7), *Torulopsis* (1), *Cryptococcus* (1) and *Sporobolomyces* (1). Four new species have been described, *Pichia silvestris*, *Pichia quercibus*, *Pichia carsonii* and *Debaryomyces fluxorum*. *P. quercibus* and *P. carsonii* are unusual representatives of the genus *Pichia* in that they form a very primitive pseudomycelium and lack pellicles on malt extract. To accommodate such species an amended diagnosis of the genus *Pichia* has been proposed by PHAFF in an accompanying paper. *Saccharomyces pastori*, of which 12 isolates were obtained, has been transferred to the amended genus *Pichia* as *P. pastori* (Guilliermond) nov. comb.

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FORMATION OF FORMALDEHYDE AND FORMYL- COMPOUNDS DURING THE RESPIRATION OF ACETATE AND GLYCOLATE BY *E. COLI*

by

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In a preceding paper by BOLCATO and SCEVOLA (1953b) the formation of benzaldehyde during the oxidative dissimilation of acetate in presence of phenylhydrazine by *E.coli* was briefly mentioned. In view of the hypothesis formulated by the same authors (1953a) to explain the biosynthesis of acetophenone observed under the same experimental conditions, it was initially supposed that during the respiration of acetate an active formate was formed which would formylate the benzene ring of phenylhydrazine to form benzaldehyde. When, however, it was observed that benzaldehyde could be formed in the absence of any microbial cells merely by letting react dilute solutions of formaldehyde and phenylhydrazine, BOLCATO and SCEVOLA (1953c) and BOLCATO (1954) suggested that benzaldehyde isolated during the oxidative breakdown of acetate in the presence of phenylhydrazine might have been formed non-enzymatically from a reaction between formaldehyde, intermediate in the process, and phenylhydrazine present in the medium.

The uncertainty as to the origin of benzaldehyde has led us to a further study of this interesting problem. The results reported in the present paper demonstrate that actually benzaldehyde originates from an enzymatic formylation of the benzene ring of phenylhydrazine. The direct isolation of formaldehyde both from acetate and glycolate oxidation by *E.coli* is also reported.

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EXPERIMENTAL.

Experiments with acetate. The avirulent C-14 strain of *E. coli* was used in all experiments. The growth on a freshly prepared slant was transferred to 350 ml of 0.6% nutrient broth (Difco) and incubated overnight at 35°C. From this medium a volume of 5 liters of a medium was inoculated containing per liter: H₂O 800 ml, yeast extract 200 ml, Na₂HPO₄ 5 g, KH₂PO₄ 2.5 g, MgSO₄·7H₂O 0.3 g, beef extract (Difco) 2.5 g, sodium acetate 6 g, glucose 2 g; pH 7. After incubation for 20 hrs with constant aeration the cells were harvested by centrifugation, washed with the following medium (medium A): H₂O 800 ml, yeast extract 200 ml, Na₂HPO₄ 2.5 g, KH₂PO₄ 1 g, (NH₄)₂SO₄ 1.5 g, MgSO₄·7H₂O 0.3 g, beef extract (Difco) 0.5 g, sodium acetate 6 g, pH 7 and immediately suspended in 1200 ml of the same medium. The suspension was transferred to a 1500 ml glass cylinder provided with a sintered diffuser for aeration. Depending on the pH changes three portions of 0.4 ml of phenylhydrazine acetate were added (Table 1). Phenylhydrazine acetate was freshly prepared by dissolving 1 g of phenylhydrazine in 50 ml of distilled water containing 0.35 g acetic acid and adjusting the pH with NaOH (pH = 7).

After a further incubation of 8-10 hrs the medium was centrifuged and used for the analysis of formed products.

Experiments with glycolate. The same technique was used for the experiments on glycolate oxidation; 0.1 g% of glycolic acid (Merck) was dissolved in medium A and neutralized with NaOH to pH 7. As soon as the respiration started 0.2 g of phenylhydrazine oxalate, which had been dissolved in 25 ml of hot distilled water and NaOH, was added depending on the pH changes of the incubation mixture as indicated in Table 1. After a further 8-10 hrs of incubation the suspension was centrifuged and analyzed.

Distillation of the medium. To separate the volatile products formed during the respiration of acetate (or glycolate) the medium was steam distilled after a previous acidification with sulphuric acid up to a concentration of 1% in order to set free the products from the phenylhydrazine. The distillation was carried out at constant volume and continued until a sample of the distillate gave no turbidity with some drops of sulphuric acid solution of 2,4-dinitrophenylhydrazine (2,4-DNP). From 500 ml of the medium about 150 ml of distillate was collected.

TABLE 1.
Oxidation of acetate and glycolate by *E. coli*.

Acetate			Glycolate		
time	pH	phenylhydrazine acetate g	time	pH	phenylhydrazine oxalate g
9.10	6.9		11.00	7.0	
11.00	7.2	0.4	13.00	7.2	0.2
14.30	7.4	0.4	14.50	7.4	0.2
15.00	7.7	0.4	15.20	7.6	0.2
17.30	8.1		15.50	7.8	0.2
19.00	8.3		16.20	8.0	0.2
			17.20	8.3	
			19.30	8.1	

Isolation of benzaldehyde. A solution of 2,4-DNP was added to the distillate, the mixture allowed to stand at 30°C. for 15 hrs and filtered. The precipitate was dried and treated with boiling 95% ethanol to separate the insoluble acetophenone- and benzaldehyde-2,4-DNP from formaldehyde- and acetaldehyde-2,4-DNP, both soluble in this solvent. The separation of benzaldehyde-2,4-DNP from acetophenone-2,4-DNP was performed according to the method previously used (1953b).

Isolation of formaldehyde. Formaldehyde was identified by two methods: 1) with the chromotropic reagent on 0.5 ml of the distillate of the medium according to the procedure used by EDWARDS and KELLIE (1954).

Under the same conditions distillation of benzaldehyde-, acetophenone-, acetaldehyde-phenylhydrazone dissolved in the medium A gives no specific colour with the chromotropic reagent.

2) Isolation of the 2,4-dinitrophenylhydrazone from the ethanolic extract obtained during the separation of the benzaldehyde-2,4-DNP (see above). This extract was evaporated to dryness and the residue treated with 20-30 ml of benzene containing 10% of ethyl acetate. After filtration the solvent was chromatographed in a Brockmann's alumina column to separate formaldehyde-2,4-DNP from red products and from any acetaldehyde-2,4-DNP which might be present. Development of the chromatogram was carried out with 5% ethylacetate in benzene. Acetaldehyde-2,4-DNP moved faster than formaldehyde-2,4-DNP whereas the red products

remained on top of the column. The band of the formaldehyde-2,4 DNP was eluted with ethyl ether. After evaporation of the solvent the product recrystallized from ethyl alcohol melted at 165°C. and no depression was observed on admixture with synthetic formaldehyde-2,4-DNP. Nitrogen content for $C_7H_6O_4N_4$ calc.: N 26.66%; found 26.58%; m.p. 165°C.

Blank tests have demonstrated that this method gives a yield of about 20% of formaldehyde present in the medium. In fact it is known that the quantitative distillation of small amounts of this aldehyde is difficult and seldom attained.

The results of 4 typical experiments are summarized in Table 2.

TABLE 2.

Formation of acetophenone, benzaldehyde and formaldehyde from the respiratory breakdown of acetate and glycolate by *E.coli* in presence of phenylhydrazine.

Media tested	Control without phenylhydrazine	Acetate with phenylhy- drazine acetate		Glycolate with phenylhy- drazine oxalate	
	A	B	C	D	E
pH initial	7.0	6.8	7.0	7.0	6.9
pH final	8.8	8.3	7.9	8.2	8.1
intake of acetic acid in %	60	45	42	—	—
acetophenone as 2,4-DNP mg/l	0.0	120	70	—	—
benzaldehyde as 2,4-DNP mg/l	0.0	45	50	105	73
formaldehyde as 2,4-DNP mg/l	0.0	3	5	7	4

The failure to trap larger quantities of formaldehyde is certainly due to the fact that the aldehyde, even when trapped as phenylhydrazone is attacked by *E. coli*, while in presence of an excess of phenylhydrazine the phenylhydrazone is transformed into benzaldehyde (Table 3, Exp. A and B).

Formylation of the benzene ring of phenylhydrazine. Two hypothesis have been formulated to explain the formation of benzaldehyde during the respiration of

acetate: 1) the aldehyde might arise from an enzymatic formylation of the benzene ring of phenylhydrazine; 2) or from a purely chemical phenylation of the intermediate formaldehyde by means of the phenylhydrazine present in the medium according to the following general equation of the phenylation reaction of aliphatic aldehydes:



To test which of these hypothesis might better explain the formation of benzaldehyde in our experiments we have submitted 0.1 g of formaldehyde with phenylhydrazine in excess or in equimolecular amount to the action of *E. coli*. The results of this experiment were compared with those obtained by purely chemical means, *viz.* by mixing formaldehyde and phenylhydrazine in the same ratio, but in the absence of microbial cells. The data on the formation of the benzaldehyde in both experiments are presented in Table 3.

TABLE 3.

Formation of benzaldehyde in the presence and in absence of *E. coli*.

mg/l	A	B	C	D
benzaldehyde as 2,4-DNP	5	120	18	10
formaldehyde as 2,4-DNP	trace	—	—	—

1000 ml of aerated *E. coli* cells suspended in the medium A with:

- A) 0.1 g formaldehyde and 0.45 g phenylhydrazine both added at the start.
 B) 0.1 g formaldehyde and 0.45 g phenylhydrazine; 0.5 g phenylhydrazine oxalate was added at the start and 0.5 g after 1 hr.

1000 ml of the medium A with:

- C) 0.1 g formaldehyde and 0.45 g phenylhydrazine.
 D) 0.1 g formaldehyde, 0.45 g phenylhydrazine and 1 g phenylhydrazine oxalate.

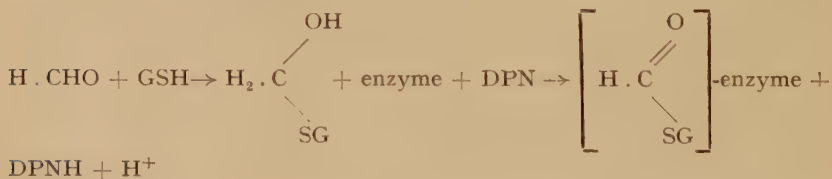
Incubation and aeration of all experiments 12 hrs, pH 5.5, temperature 30°C.

Formaldehyde in the presence of an equimolecular amount of phenylhydrazine (i.e. in the form of phenylhydrazone) was rapidly oxidized by the cells and benzaldehyde was formed in small quantity (Table 3, Exp. A). Only in the presence of an excess of phenylhydrazine benzaldehyde was formed with good yield: 120 mg/l as 2,4-DNP (Exp. B). In the absence of *E. coli* (Exp. C and D) the formation of benzaldehyde, according to equation I, when compared with the biochemical reaction gave a lower yield when phenylhy-

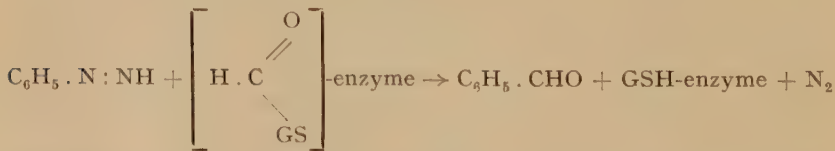
drazine was in excess and a higher one when phenylhydrazine was in equimolecular ratio with formaldehyde.

It has been reported in another paper (in press) that the purely chemical phenylation of formaldehyde resulting with the formation of benzaldehyde is a reaction which occurs at slow rate, with low yield, better under anaerobic conditions and with an equimolecular amount of phenylhydrazine. On the contrary, the reaction catalyzed by *E.coli* is more rapid, giving a greater yield and requiring aerobic conditions and an excess of phenylhydrazine. From these results we may conclude that the chemical and biochemical reactions which lead to the same final product are two independent reactions working with different mechanisms.

For the biochemical reaction it has been supposed that phenylhydrazine might be dehydrogenated to give rise to an unstable intermediate, probably the di-imide: $C_6H_5.N:NH$. Nitrogen might be split off giving rise to an active phenyl which would be immediately formylated by a formyl group arising from the intermediate formaldehyde. In this connection it may be mentioned that recently STRITTMATTER and BALL (1955) have isolated from chicken and beef liver a specific DPN-dependent formaldehyde dehydrogenase with glutathione as a specific co-factor. Assuming that glutathione (GSH) participates directly in this reaction these authors have postulated a mechanism for the oxidation of formaldehyde which includes the following reaction:



The formyl group, formed at level of the dehydrogenase system, could formylate the benzene ring of phenylhydrazine, arising from the di-imide, to form benzaldehyde:



BEAVEN and WHITE (8) have found that oxyhaemoglobin can act on phenylhydrazine yielding nitrogen and benzene, probably via

the di-imide. This result is an additional evidence for the supposed formation of an active group in our experiments.

In the experiments on acetate oxidation by *E. coli*, acetophenone is always formed in greater amount than benzaldehyde (1953a). In the case of acetate oxidation by yeast cells acetophenone is a result of acetylation of the benzene ring of phenylhydrazine, probably by means of the intermediate formation of the complex acetyl-CoA (BOLCATO, 1956).

The suggestion that acetophenone might also be formed by a purely chemical phenylation of the intermediate acetaldehyde (BOLCATO and SCEVOLA, 1953c) might be incorrect.

CONCLUSION.

The results of our experiments demonstrate that formaldehyde and the formyl group are common intermediates in the acetate and glycolate oxidation. This fact provides evidence for the following sequence of reactions in *E. coli* cells:



The possible formation of glycolate from acetate has been observed by CHALLENGER *et al.* (1927) and by BERNHAUER and SCHEUER (1932) in experiments with *Aspergillus niger*. WEINHOUSE (1951), working with the same mold with labeled acetate in the presence of unlabeled glycolate, has found the isolated glycolic acid appreciably radioactive. Further investigations are in progress to establish whether the results of our respiration experiments agree with the hypothesis of NORD and VITUCCI (1948), that acetate oxidation in microbial cells might occur via glycolic acid.

S u m m a r y.

Acetate and glycolate respiring cells of *E. coli* are able to formylate the benzene ring of phenylhydrazine to form benzaldehyde. In both cases it was also possible to isolate a little formaldehyde.

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NITRATE REDUCTION IN THE GENUS *CHROMOBACTERIUM*

by

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(Received October 15, 1955).

More than 80 cultures of violet chromogens have been collected during the past five years with a view toward developing a better classification of this genus. Some were isolated from soil by the method of CORPE (1951) but the largest number were obtained from individuals and Type Culture Collections. These came from widely separated areas and were isolated from diverse sources.

It has long been known that strains of this genus are difficult to keep alive without frequent transfer and even then many are lost when kept in refrigerators. This has been one reason why this genus has not been studied adequately. Two sets of cultures were put under sterile mineral oil to a depth of one centimeter above the top of the agar. One set was kept on peptone beef extract agar and the other on 1 % Difco Bacto peptone agar. In most cases the pigment was better without the beef as was also found by CORPE (1953). Most strains could be kept in this way at room temperature for a year or more without transfer. The more viscous the growth the less well the organism survived under oil. It was therefore, found advisable to keep a third set at room temperature with transfer every four weeks.

During a preliminary study of the cultural characteristics of the various strains, it was noticed that when Levine's nitrate broth (PELTIER *et al.* 1952) was used to test nitrate reduction, growth was irregular and variation among strains appeared which did not agree with other characteristics described by CRUESS-CALLAGHAN and GORMAN (1935) which are quoted in BERGEY'S Manual (1948). As the oxygen tension of a medium has been reported by SACKS and

BARKER (1949) and CASTILOW and HUMPHREYS (1955) to be an important factor in the ability of an organism to reduce nitrate, it was thought that a comparison of strains of *Chromobacterium* using different types of nitrate media containing small amounts of agar might show even more variation between strains.

MATERIALS AND METHODS.

The media chosen for this comparison were ZoBell's, ZoBell's with potassium nitrite instead of nitrate, Levine's with 0.3 % agar and 0.5 % glucose added, B.B.L. Indol Nitrite and Difco nitrate broth with the addition of 0.3 % yeast extract and 0.1 % agar as used by CASTILOW and HUMPHREYS (1955).

All media were tubed in 10 ml amounts and kept at room temperature after sterilization. Tubes were inoculated in duplicate with a 24 hour peptone broth culture of the different strains and incubated at 25-27°C. They were examined for growth and gas production at the end of 24 and 48 hours and were tested for nitrite and nitrate at the end of 2, 7, 10, and 14 days using the tests recommended in the Manual of Methods for Pure Culture Study of Bacteria (1946) with the exception that for all tests except the final one, porcelain plates were used to test one 4 mm loop of each culture. This experiment was repeated with all media three times. A fourth set of tubes were incubated at 28-29°C. instead of at 25-27°C.

RESULTS AND DISCUSSION.

Table 1 gives a summary of the results in the media used. It may be noted that the stage to which nitrate was reduced was often dependent upon the composition of the medium. The presence of glucose with peptone seemed to reduce or prevent complete reduction by some strains. The semisolid medium of ZoBell and the Difco medium with added yeast and agar gave complete reduction of nitrate by the largest number of strains. So far as known CORPE (1954) who reported gas produced by some of his soil strains, is the only person who has indicated that strains of this genus could reduce nitrate beyond nitrite, though whether or not nitrate was reduced to nitrite was used by CRUESS-CALLAGHAN and GORMAN (1935) as a characteristic in separating species.

The rate at which nitrate was completely reduced depended upon the strain. D3, 40, 41, Mg2.2, 10,57 and 18 reduced rapidly with

TABLE 1.
Products of nitrate reduction by cultures of *Chromobacterium*.

Culture No.	Levine's Broth	Levine's plus 0.3% agar and 0.5% glucose	ZoBell's Medium	BBL Indol Nitrite	Difco Nitrate plus 0.3% yeast and 0.1% agar	ZoBell's with nitrite
	NO ₂	gas NO ₂ NO ₃	gas NO ₂ NO ₃	gas NO ₂ NO ₃	gas NO ₂ NO ₃	gas NO ₂
1, 2, 3	+	— +	+ — —	— +	— — —	± —
9,30	+	— +	— — —	— +	— — —	— —
10	+	— +	+ — —	+ — —	+ — —	+ —
32 1)	+	— +	— — —	— +	+ — —	— —
40, 41, D3	—	+ — —	+ — —	+ — —	+ — —	+ —
7917	—	— +	— — —	— +	+ — —	— —
18	+	— +	+ — —	+ — —	+ — —	— —
21	+	— +	— — —	— — —	— — —	— —
22	+	— +	+ — —	— — —	— — —	— —
3434, 6915	+	— +	— + —	— + —	— ± —	— —
11104	+	— +	+ — —	— + —	+ — —	+ —
57	+	— — —	+ — —	+ — —	+ — —	+ —
59, K2, 7150	+	— +	— + —	— + —	+ — —	— —
60	—	— +	— — —	— — —	— — —	— —
61, L674	+	— +	+ — —	— — —	— — —	— —
71	+	— +	— + —	— + —	— — —	— —
75	+	— +	+ — —	— + —	— — —	— —
Mg 2.2	+	— +	+ — —	+ — —	+ — —	+ —
B-1020 2)	+	— — +	— ± +	— — +	— ± —	— ±
101	+	— +	+ — —	— + —	+ — —	+ —
106	+	— — —	— — —	— — —	— — —	— —
Mg, 2.1	—	— +	+ — —	— +	+ — —	— —
4 3)	+	— +	— +	— +	— +	— +
23 4)	—	— — +	— — +	— — +	— — +	— +

1) produced gas only when incubated at 28-29°C.

2) very slow to reduce both nitrate and nitrite. Results vary.

3) like results for strains 5, 6, 7, 8, 11, 12, 114, 14, 15, C15, 20, 25, 26, 21.251, 29, P104, 13S, 42, 44, 36a, 6357, 553, 7461, 62, 63, 64, 66, 67, 68, 69, 532, 78, 92, 93, 95, 100, 102, 103, 104, 105 and 109.

4) like results for strains 24, 13S₁, 58, B-1085, 65 and 94.

+ positive test for substances.

copious gas production, while Mg2.1, 101, 75, 1, 2, 3, and 32 reduced with gas production but much more slowly. Strains 9, 30, 21, 60 and 106 reduced all nitrate beyond nitrite but never with any visible gas. Strains 61 and L674 sometimes produced gas in ZoBell's medium but were very slow to reduce all nitrate beyond nitrite. Strain 3434 varied in its ability to reduce nitrate but was always able to reduce nitrite. All strains which reduced nitrate beyond nitrite in any of the semisolid media were able to reduce nitrite in ZoBell's medium with nitrite substituted for nitrate. KLUYVER and VERHOEVEN (1954 b) also found in their work with *Micrococcus denitrificans* Beijerinck that nitrite or even nitrous oxide could be substituted for nitrate in the denitrification process.

Denitrification with the production of gas has been observed in

only a few groups of bacteria. VERHOEVEN and GOOS (1954) collected and analyzed the gas produced in the reduction of nitrate by *Pseudomonas aeruginosa* (Schroeter) Migula and found it to be 10 % nitrous oxide. ALLEN and VAN NIEL (1952) using *Pseudomonas stutzeri* (Lehmann and Neumann) Kluyver, non Migula, found that small amounts of cyanide prevented the reduction of nitrate and nitrous oxide but not the reduction of nitrite to nitrogen and concluded that nitrous oxide is not a normal intermediate product of denitrification. KLUYVER and VERHOEVEN (1954 a) concluded from carefully controlled experiments with *Pseudomonas aeruginosa*, *P. stutzeri* and *Micrococcus denitrificans* that nitrous oxide is a normal intermediate product in nitrate reduction but they also concluded that the nitrogen produced may originate partly from a hydrogenation of nitrous oxide and partly from a direct hydrogenation of the precursor of nitrous oxide.

The 80 strains of *Chromobacterium* studied belong in three groups: I. those which completely reduce nitrate with or without gas production; II. those which reduce to nitrite only; III. those which do not reduce nitrate.

Group I.

This group includes strains 1, 2, 3, 9, 30, 10, 32, 40, 41, D3, 7917, 18, 21, 22, 3434, 6915, 11104, 57, 59, K2, 7150, 60, 61, L674, 71, 75, Mg2.2, B-1020, 101, 106 and Mg2.1 which were found to reduce nitrate completely with or without gas production. These cultures came mostly from water or soil varying in locality from New Hampshire to Kentucky of northern Georgia. One came from decomposing cocoanut and another from white bread resting on soil but none were isolated from man or animals. Apparently CRUESS-CALLAGHAN and GORMAN's cultures that they identified as *Bacterium violaceum* and as *Bacterium violaceum amethystinum* would be included in this group.

Group II.

Group II is made up of strains 4, 5, 6, 7, 8, 11, 12, 114, 14, 15, C 15, 20, 25, 26, 21.251, 29, P104, 13S, 42, 44, 36a, 6357, 553, 7461, 62, 63, 64, 66, 67, 68, 69, 532, 78, 92, 93, 95, 100, 102, 103, 104, 105 and 109 and were found to reduce nitrate to nitrite only. Many were isolated from soil and water from Trinidad, Malaya, and the

southern states. It is interesting that all cultures isolated from man or warm blooded animals fell into this group. Apparently some or perhaps all of these cultures would fall in the species designated by CRUESS-CALLAGHAN and GORHAN as *Bacterium janthinum*.

Group III.

This is a small group which includes strains 23, 24, 13S₁, 58, B-1085, 65 and 94 which were unable to reduce nitrate. Their source was diverse but none came from man or warm blooded animals. These would also seem to belong to CRUESS-CALLAGHAN and GORMAN's *Bacterium janthinum*.

Based upon the relationships just discussed, at least 3 species should be recognized among the violet organisms. A further study of other characters might indicate that one or more of these 3 groups should be further sub-divided.

A c k n o w l e d g m e n t s .

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S u m m a r y .

Whether or not a strain of *Chromobacterium* is able to reduce nitrate completely depends upon the oxygen tension of the medium, the composition of the medium and the length of time the culture is incubated. Based upon action on nitrate only, the genus may be divided into three parts: those which reduce completely with or without gas production, those which reduce to nitrite only and those which do not reduce.

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THE TAXONOMY OF YEASTS ISOLATED FROM *DROSOPHILA* IN THE YOSEMITE REGION OF CALIFORNIA

by

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(Received October 1, 1955).

The present paper gives a taxonomic account of the yeast flora isolated from the intestinal canal of wild species of *Drosophila* occurring at altitudes from 4500 feet to 10,000 feet, in the Yosemite region of the Sierra Nevada in California. The relationships between these yeasts and the species of *Drosophila* from which they were obtained have been described in a separate publication (9). Besides a general discussion of the yeasts isolated, a number of new species will be described. In addition, the yeast flora found by us will be compared with the results of surveys by other investigators.

METHODS.

Methods of collecting the flies, dissection of the crops and isolation procedures have been described in detail by PHAFF, *et al.* (9). Identification was first attempted by the system of LODDER and KREGER – VAN RIJ (5) or that of WICKERHAM for *Hansenula* (17). If there was doubt about the identity of an isolate with a known species, an authentic culture was usually obtained and a detailed comparison was made. This included a study of their assimilatory ability using a large number of carbon compounds as recommended by WICKERHAM (17). If a culture could not be identified by either system or by comparison with other yeasts described in the literature, it was considered a new species. During the initial study the replica plating method was used to determine assimilation reactions (14). This method is especially useful and time saving when a large number of strains are isolated, allowing a rapid tentative grouping of the cultures. For confirmation the method of WICKERHAM (17),

using a liquid synthetic medium in test tubes, was applied to all new species and in cases of doubt. The carbon compounds used are given in Table 2. The yeasts were also tested for their ability to grow in a vitamin free medium (17) and acid production was determined after growth on chalk agar (see PHAFF and KNAPP, (10).

RESULTS.

The 241 cultures isolated are listed in Table 1. The association of the different yeasts with the various species of *Drosophila* from which they were isolated has been discussed in detail by PHAFF, *et al.* (9).

Genus *Saccharomyces*. This genus is represented by a great number of isolates. The most common yeast was considered to be a new species in our previous paper (9) and was termed provisionally *S. montanus*. Although the fermentation characteristics are the same as those of *S. cerevisiae* and the key to the species of the genus *Saccharomyces* given by LODDER and KREGER - VAN RIJ (5) leads to this species, we considered it to be different for the following reasons. Its cell dimensions are smaller than their group II (small celled group), many of the cells conjugated before sporulation and, perhaps most important of all, it assimilated many more carbon compounds than does *S. cerevisiae*. However, on rechecking some of the older species of *Zygosaccharomyces* described by STELLING-DEKKER (15), we noticed great similarities between our isolates and *Zygosaccharomyces fermentati* described originally by NAGANISHI in 1928, who isolated it from the sediment of a peppermint beverage. LODDER and KREGER - VAN RIJ placed this species in *S. cerevisiae* and considered them synonyms. We obtained an authentic transfer of the former *Z. fermentati* from the C.B.S.¹⁾ culture collection and found that our isolates were indeed remarkably similar to this yeast, including the assimilation pattern of the 34 carbon compounds used. Since there was little variation in the carbon assimilation reactions among our 36 isolates of this yeast, we believe that *Z. fermentati* is a well defined and valid species. In line with the now well accepted practice to abolish *Zygosaccharomyces* as a separate genus, the species should be termed *S. fermentati* (Naganishi) nov. comb. This, however, is not possible since LODDER and KREGER - VAN RIJ (5) transferred *Torulaspora fermentati* (Saito) to *Saccharomyces* and

¹⁾ Centraal Bureau voor Schimmelcultures, Yeast Division, Delft, Holland.

TABLE 1.

Yeasts isolated from *Drosophila* in the Yosemite Region of California.

Species of yeast	No. of isolates	Areas in which isolated ¹⁾
Sporulating yeasts		
1. <i>Saccharomyces montanus</i> (Naganishi) nov. comb.	36	M; PF
2. <i>S. veronae</i> Lodder et Kreger-van Rij 1952	30	M; AV; T
3. <i>S. cerevisiae</i> Hansen var. <i>tetrasporus</i> (Beijerinck) nov. comb.	22	M
4. <i>S. drosophilarum</i> Shehata, Mrak et Phaff 1955	13	M
5. <i>S. drosophilarum</i> Shehata, et al. var. <i>acellobiosa</i> nov. var.	2	M
6. <i>S. florentinus</i> (Castelli) Lodder et Kreger-van Rij 1938	2	M; PF
7. <i>S. dobzhanskii</i> Shehata, Mrak et Phaff 1955	1	PF
8. <i>S. wickerhamii</i> nov. spec.	2	YC; AV
9. <i>S. uvarum</i> Beijerinck 1898	1	PF
10. <i>S. kluyveri</i> nov. spec.	1	M
11. <i>Pichia fermentans</i> Lodder 1932	2	M
12. <i>Pichia xylosa</i> nov. spec.	1	M
13. <i>P. membranaefaciens</i> Hansen 1888	1	M
14. <i>P. pini</i> (Holst) Phaff 1936	1	M
15. <i>P. pastori</i> (Guilliermond) Phaff 1919	2	M
16. <i>Hansenula angusta</i> Wickerham 1951	19	M; AV; PF
17. <i>Hanseniaspora valbyensis</i> Kloecker 1912	6	M
18. <i>H. uvarum</i> (Niehaus) Shehata et al. 1932	6	M
19. <i>H. osmophila</i> (Niehaus) nov. comb. 1932	5	M
Non-sporulating yeasts		
20. <i>Kloeckera apiculata</i> (Reess emend. Kloecker) Janke 1870	15	M; PF; T
21. <i>K. magna</i> (De'Rossi) Janke 1920	13	M
22. <i>Torulopsis stellata</i> (Kroemer et Krumbholz) Lodder 1931	10	M; PF; T
23. <i>T. glabrata</i> (Anderson) Lodder et de Vries 1917	1	AV
24. <i>T. pinus</i> Lodder et Kreger-van Rij 1952	1	AV
25. <i>T. inconspicua</i> Lodder et Kreger-van Rij 1952	1	AV
26. <i>T. colliculosa</i> (Hartmann) Saccardo 1903	1	M
	195	

¹⁾ Areas studied are indicated as follows: M = Mather area; AV = Aspen Valley; SJ = Smoky Jack; YC = Yosemite Creek; PF = Porcupine Flat; T = Tioga Pass area. For a more detailed description of the areas indicated, see COOPER, D. M. and DOBZHANSKY, TH. 1956. Ecology (in press).

TABLE 1 (continued).

Species of yeast	No. of isolates	Areas in which isolated
	195	
27. <i>Cryptococcus diffluens</i> (Zach) Lodder et Kreger-van Rij 1934	5	M; YC; T
28. <i>Cr. laurentii</i> (Kufferath) Skinner 1920	2	M; PF
29. <i>Rhodotorula aurea</i> (Saito) Lodder 1934	1	PF
30. <i>Rh. minuta</i> (Saito) Harrison 1922	1	AV
31. <i>Rh. glutinis</i> (Fres.) Harrison 1852	1	M
32. <i>Rh. mucilaginoso</i> (Jörg.) Harrison 1909	1	PF
33. <i>Candida krusei</i> (Cast.) Berkhout 1910	10	M; AV
34. <i>C. catenulata</i> Diddens et Lodder 1942	6	AV
35. <i>C. parapsilosis</i> (Ashf.) Lang. et Talice var. <i>intermedia</i> Kreger-van Rij et Verona 1949	6	SJ
36. <i>C. parapsilosis</i> (Ashf.) Lang. et Talice 1928	2	M; AV
37. <i>C. mesenterica</i> (Geiger) Diddens et Lodder 1910	4	M; PF; T
38. <i>C. mycoderma</i> (Reess) Lodder et Kreger-van Rij 1870	2	M; PF
39. <i>C. guilliermondii</i> (Cast.) Lang. et Guerra 1912	1	M
40. <i>Trichosporon aculeatum</i> nov. spec.	2	AV; T
41. <i>Tr. fermentans</i> Diddens et Lodder 1942	1	M
42. <i>Oospora lactis</i> (Fres.) Saccardo	1	M
Total number of isolates	241	

retained the name *fermentati*, as they did not recognize NAGANISHI's yeast as a valid species. Furthermore SAITO's *Torulaspora fermentati* or *Saccharomyces fermentati* (if one considers the genera synonymous) has priority since it was described in 1923, five years before NAGANISHI described his organism. To avoid further confusion it is proposed to change the name *Zygosaccharomyces fermentati* Naganishi to *Saccharomyces montanus* Naganishi nov. comb. A comparison of the carbon assimilation reactions of this yeast and *S. cerevisiae* is given in Table 2.

Another very common species is *Saccharomyces veronae* of which 30 isolates were obtained. This organism was originally termed *Zygosaccharomyces drosophilae* by SHEHATA and MRAK (11) although these authors did not describe it as a new species at that time. In 1952 LODDER and KREGER-VAN RIJ (5) described a new species, *S. veronae*, which was isolated by VERONA from grapes in Italy.

SHEHATA *et al.* in a later paper (13) recognized the identity of the organisms isolated from *Drosophila* with *S. veronae*.

The next frequent organism (22 isolates) is designated as *S. cerevisiae* var. *tetrasporus*. Our cultures appear to be quite similar to the yeast which BEIJERINCK isolated from the exudate of an oak. He named it *S. tetrasporus*, although he did not describe it. STELLING-DEKKER (15) who studied this organism, named it *S. mangini* var. *tetrasporus*, based on an erroneous observation that maltose was not fermented (5, pg. 128). This error was probably based on a delayed fermentation of maltose, which we also frequently have observed in our strains. Other typical features of our isolates are the very high percentage of sporulating cells on malt agar and the small size of the cells as compared to a standard culture of *S. cerevisiae*. These properties agree well with the description given by STELLING-DEKKER and we feel that the cultures are sufficiently distinct to separate them as *S. cerevisiae* var. *tetrasporus*. The carbon assimilation patterns of our isolates were all alike and they resemble *S. cerevisiae* in that respect except that α -methyl-D-glucoside was used regularly by the variety but not by a group of isolates of bakers yeast studied by us (see Table 2).

S. drosophilorum (13 isolates) has been described recently by SHEHATA, MRAK and PHAFF (13). It is a yeast with kidney- or crescent-shaped ascospores. We isolated two cultures which resembled *S. drosophilorum* a great deal. The principal difference is that these two isolates can not attack cellobiose or salicin and they therefore appear to lack a functional beta-glucosidase. Since they are otherwise very similar we will tentatively designate them as *S. drosophilorum* var. *acellobiosa*, rather than create a new species. Pending further studies regarding the relationship between the species and the variety a description of the variety will not be given at this time.

Two cultures were obtained which corresponded very well to *S. florentinus*, a small-celled, melibiose fermenting yeast, originally isolated by CASTELLI from grape must treated with sulfur dioxide. Our cultures sporulated after 4 days on malt extract. Asci were formed after isogamic conjugation or without conjugation. According to LODDER and KREGER - VAN RIJ (5) the type species (only one strain has been isolated) lost the ability to sporulate. A comparison of the carbon assimilation pattern of our strains with that of the authentic culture proved to be the same. It is given in Table 2.

Saccharomyces dobzhanskii, a yeast with kidney-shaped ascospores,

was recently described by SHEHATA *et al.* (13). These authors isolated a single strain in the Southern California mountains. A single culture was also isolated in the present survey. The two cultures agreed in all respects.

Saccharomyces wickerhamii, another yeast with kidney- to crescent-shaped ascospores, was isolated twice and must be considered a new species since both the fermentation and assimilation characteristics are different from any known species. It will be named after Dr L. J. WICKERHAM in recognition of his extensive contributions to the taxonomy of yeasts.

Standard description of *Saccharomyces wickerhamii* nov. spec.

Growth in malt extract: After 2 days at 25°C. cells are oval to slightly elongate $(2.1 - 5.2) \times (3.1 - 8.7) \mu$, occurring singly, in pairs and in branched chains. A slight sediment is present but no ring or pellicle. In older cultures a spotty ring develops, occasionally islets.

Growth on malt agar: After 1 month the growth is yellowish-buff. The surface is slightly glossy, smooth with an indication of sectoring at the edge. Texture butyrous. Cross section of the streak is almost flat. The margin is slightly irregular but not hairy.

Slide cultures: A pseudomycelium was not present after 10 days.

Sporulation: Abundant on malt agar in 3 days. Spores kidney- to crescent-shaped, usually 4 per ascus. Conjugation prior to ascus formation has not been observed. The asci rupture very soon after maturation.

Fermentation: glucose +, galactose + (weak), sucrose + (slow). Maltose, raffinose and lactose are not fermented.

Assimilation: glucose, galactose, L-sorbose (latent), sucrose, cellobiose, lactose, D-xylose, ethanol (with the formation of a thin, smooth pellicle), glycerol, D-sorbitol (latent and weak), salicin, lactic acid and succinic acid (weak). The other compounds are not utilized. Arbutin is split weakly.

Assimilation of KNO_3 : negative.

Acid production on chalk agar: weak.

Growth in a vitamin free medium: absent.

The cultures were isolated from *Drosophila montana* and *D. pinicola* in two different areas. Culture F-394A has been designated as the type species.

One culture was isolated which could be identified with *Sacharomyces uvarum*. An authentic culture of this melibiose fermenting yeast was obtained from the C.B.S. culture collection and its assimilation pattern proved to be identical with that of our strain.

Since the complete assimilation reactions have not been reported in the literature they are given in Table 2.

Another melibiose fermenting yeast was isolated which could not be identified with previously described species. It resembles *S. microellipsodes* in its fermentation characteristics, except that galactose is fermented strongly. Furthermore it can assimilate maltose, ethanol, D-sorbitol, D-mannitol and α -methyl-D-glucoside, which cannot be used by *S. microellipsodes*. We propose to name the new yeast in honor of Professor A. J. KLUYVER in recognition of his stimulation of the many taxonomic studies on yeast carried out in his laboratory.

Standard description of *Saccharomyces kluyveri* nov. spec.¹⁾.

Growth on malt extract: After 3 days at 25°C. cells oval to long-oval $(2.3 - 7.0) \times (2.6 - 11.5) \mu$, occurring singly, in pairs and in well developed chains. Only a sediment is present. In older cultures a fairly well developed ring develops gradually, but a pellicle is not formed.

Growth on malt agar: After one month the growth is cream colored. Center of the streak has a few craters, but the surface is otherwise smooth and semi-glossy. Texture is butyrous. The cross-section of the streak is convex. The margin is somewhat irregular with an indication of a few tufts of pseudomycelium.

Slide cultures on potato dextrose agar: A few tufts of primitive pseudomycelium were observed, particularly underneath the coverslip. There is little differentiation between pseudomycelium cells and blastospores.

Sporulation: spores are formed abundantly after 12 days on vegetable agar. Nearly always 4 spherical spores per ascus and there is no evidence of conjugation prior to spore formation. The spores have a smooth surface and contain a small refractive granule. The asci do not rupture at maturity.

Fermentation: glucose +, galactose +, sucrose +, raffinose + (complete). Maltose and lactose are not fermented.

Assimilation: glucose, galactose, L-sorbose (latent and weak), maltose, sucrose, melibiose, raffinose, melezitose (latent and weak), ethanol (with the production of a slight pellicle), D-mannitol, D-sorbitol and α -methyl-D-glucoside. Lactic and succinic acid appear to be utilized very weakly. Arbutin is not split. The other compounds are not assimilated.

Assimilation of KNO_3 : negative.

Acid production on chalk agar: rather weak.

Growth in a vitamin free medium: absent.

The culture was isolated from *Drosophila pinicola* at Mather.

¹⁾ A second strain of this species was recently isolated in our laboratory from the exudate of a willow in Davis, California.

TABLE 2.

Assimilation data for some yeasts isolated from *Drosophila*. V indicates variable results (some strains assimilate the compound, others do not or only weakly). L indicates latent utilization. W signifies weak growth.

	D-glucose	D-galactose	L-sorbose	Maltose	Sucrose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	Melezitose	Inulin	Soluble starch	D-xylose	L-arabinose	D-arabinose	D-ribose
<i>S. montanus</i>	+	+	+	+	+	+	+	—	—	+	+	+	—	—	+	—	—
<i>S. cerevisiae</i>	+	+	—	+	+	—	L	—	—	+	+	—	—	—	+	—	—
<i>S. cerevisiae</i> var. <i>tetrasporus</i>	+	+	—	+	+	—	V	—	—	+	+	—	—	—	—	—	—
<i>S. florentinus</i>	+	+	+	+	+	—	+	—	+	+	+	—	—	—	+	—	—
<i>S. uvarum</i>	+	+	—	+	+	—	+	—	+	+	+	—	—	—	+	—	—
	L-rhamnose	Ethanol	Glycerol	i-Erythritol	Adonitol	Dulcitol	D-mannitol	D-sorbitol	α-methyl-D-Glucoside	Salicin	Gluconic acid	Ca-2-keto-gluconate	K-5-keto-gluconate	D-L lactic acid	Succinic acid	Citric acid	i-Inositol
<i>S. montanus</i>	—	W	—	—	—	—	+	+	+	+	—	+	—	+	+	—	—
<i>S. cerevisiae</i>	—	V	L or	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>S. cerevisiae</i> var. <i>tetrasporus</i>	—	— or	—	—	—	—	V	—	—	—	L or	—	—	—	—	—	—
<i>S. florentinus</i>	—	W	—	—	—	—	+	+	+	—	W	—	W	+	+	—	—
<i>S. uvarum</i>	—	—	V	—	—	—	—	—	+	—	—	—	—	—	—	—	—

Genus *Pichia*. Seven isolates were obtained belonging to *Pichia*. The two cultures of *P. fermentans* and the one of *P. membranaefaciens* fitted the standard descriptions very well. Complete carbon assimilation reactions of these cultures were not determined, since the isolates were lost during the early phases of the investigations. The fourth culture does not form a pellicle on malt extract, although it produces a well developed pseudomycelium. It fits in the amended diagnosis of the genus *Pichia* given in the preceding paper (10a). Since the carbon assimilation reactions are quite different from previously described species it is considered to be a new species. It has been named *Pichia xylosa*, since it utilizes the sugar D-xylose in contrast to most species of this genus.

Standard description of *Pichia xylosa* nov. spec.

Growth in malt extract: After 3 days at 25°C. cells short-oval, $(2.1 - 4.9) \times (3.5 - 7.0) \mu$, occurring singly, in pairs and in clusters up to about nine cells. A spotty ring begins to form and a sediment, but no film. After 3 weeks a heavy ring and sediment are present and a few islets, which may have formed from fragments of the ring.

Growth on wort agar: After 1 month whitish to cream colored,

semi-glistening with some craters and sectors on the surface. Texture butyrous. Cross section rather low convex. Margin irregular with a very short fringe of pseudomycelium.

Slide culture on potato-dextrose agar: After one week a well developed pseudomycelium present. Aerobically the *Blastodendron* type is typical whereas anaerobically (under the coverslip) a *Mycotoruloides* type is more common.

Sporulation: Fairly abundant on malt agar after 10 days. Spores hat-shaped, 2-4 per ascus (mostly four). Conjugation prior to sporulation was not observed. The asci rupture readily at maturity. Spores contain a small refractive granule.

Fermentation: Only glucose is fermented (slowly). A full tube of gas is produced in about 10 days.

Assimilation: Glucose, maltose, sucrose, cellobiose, trehalose, D-xylose, L-rhamnose (weak), ethanol, glycerol, D-mannitol, D-sorbitol, salicin, gluconic acid, lactic acid, succinic acid and citric acid. Arbutin is split. The other compounds are not utilized.

Assimilation of KNO_3 : negative.

Growth in vitamin free medium: negative.

Acid production on chalk agar: rather weak.

The remaining 3 isolates were designated as *Saccharomyces pastori* (2 cultures) and *S. pini* (1 culture) in our previous paper (9). In line, however, with the arguments presented by PHAFF (10a) who favored a transfer of these two species to the amended genus *Pichia*, these yeasts will be considered as *Pichia pastori* and *Pichia pini* respectively.

Genus *Hansenula*. *H. angusta* was the only species isolated and it is represented by 17 isolates. Their properties corresponded quite well with the description given by WICKERHAM, except that our isolates were unable to utilize D-arabinose and D-xylose, which are reported to be variable or positive for the type species. This property of our strains has been confirmed by Dr WICKERHAM and it is therefore best to consider the utilization of these compounds as variable. Otherwise the cultures are quite similar. *H. angusta* appears to have its natural habitat in association with insects in deciduous trees (WICKERHAM, personal communication) and it seems likely that the flies obtained the yeast from such trees. It is interesting, however, that this yeast was not isolated from the fluxes of oaks (10) which represent the principal deciduous tree in the areas indicated in Table 1.

Genus *Hanseniaspora*. Three species were found, *H. valbyensis* (6 isolates), *H. uvarum* (6 isolates) and *H. osmophila* (5 iso-

lates). The last two species may also be considered as members of the genus *Kloeckeraspora* Niehaus. However, we have followed SHEHATA *et al.* (13) who proposed to transfer the species of this rather doubtful genus to *Hanseniaspora*. *H. uvarum* and *H. osmophila* have retained their ability to sporulate fairly well over a period of 3 to 4 years. NIEHAUS (7) reported that his strains lost this ability soon after isolation. We usually found one spore per ascus, occasionally two. The two species are differentiated by the fact that *H. osmophila* assimilates maltose, whereas *H. uvarum* cannot utilize this sugar.

Genus *Kloeckera*. This genus is generally considered the imperfect form of *Hanseniaspora* although not all species of *Kloeckera* have known perfect forms. Both *Kloeckera apiculata* (15 isolates) and *Kloeckera magna* (13 isolates) were quite common. We agree with LODDER and KREGER - VAN RIJ that *K. apiculata* is the imperfect form of *Hanseniaspora valbyensis*. *Kloeckera magna* is probably the imperfect form of *H. osmophila*. It is interesting that we isolated both the perfect and the imperfect forms of these two yeasts in our survey. We have confirmed the earlier observation of WICKERHAM and BURTON (16) that cellobiose is always among the very few carbon compounds which the apiculate yeasts can utilize. Some of our strains were able to cause a weak fermentation of cellobiose. It seems likely that these yeasts have a competitive advantage in the forest, by being able to use this breakdown product of cellulose produced by extracellular enzymes of other organisms.

Genus *Torulopsis*. Of the 5 species of *Torulopsis* only *T. stellata* was found in significant numbers (10 isolates from 3 areas). According to LODDER and KREGER - VAN RIJ (5) the C.B.S. collection has only one strain, isolated in 1931 from "Trocken-beerenauslese" by KROEMER and KRUMBHOLZ (2,3). This material consists of grapes with a very high sugar content shriveled on the vine. It is of interest that isolates of this species die rather rapidly on malt-agar slants. After 4 to 6 months the streak cultures are quite brown and very few cells are viable. *Torulopsis glabrata* has been obtained usually from human sources and appears to be associated with certain infections. The first culture from other sources was reported by PHAFF *et al.* (8) who isolated it from shrimp caught in the Gulf of Mexico. The single isolate from *Drosophila* is another example of a non-pathological origin. All isolates belonging

to *Torulopsis* corresponded well with the descriptions given by LODDER and KREGER-VAN RIJ.

Genus *Cryptococcus*. Five isolates were found to be similar to *Cr. diffluens* and two to *Cr. laurentii*. All produced starch-like compounds and contained very low concentrations of carotenoid pigments, which fits the definition of the genus. It might be mentioned that the species of this genus, as defined by LODDER and KREGER-VAN RIJ (5), constitute mixtures of different metabolic types. The reason is that these authors used only five sugars in differentiating these non-fermentative yeasts. If more carbon compounds are used large differences between strains of the present species can be detected. Already the addition of raffinose and melibiose to the customary five sugars shows up distinct differences. At this time, however, we do not wish to create a number of additional species on this basis in *Cryptococcus* (or in other imperfect genera as well) until such genera can be studied in detail. In addition the taxonomic position of the genus *Cryptococcus* has been somewhat weakened by the studies of NAKAYAMA *et al.* (6) who showed that strains of certain species of *Cryptococcus* were able to produce appreciable quantities of β -carotene, giving the cultures a yellow appearance.

Genus *Rhodotorula*. In line with the above statements we have decided to retain the species *Rhodotorula aurea* (Saito) Lodder in the genus *Rhodotorula* rather than consider it as a synonym of *Cr. laurentii* as was done by LODDER and KREGER-VAN RIJ (5). One isolate was obtained which corresponded exactly to the description given by LODDER (4). Although it produces a starch-like compound, the isolate was distinctly orange-yellow on malt extract slants and significant quantities of carotenoid pigments could easily be demonstrated in this yeast by the procedure of NAKAYAMA *et al.* (6). In this respect one might say that this yeast occupies an intermediary position between *Cryptococcus* and *Rhodotorula* as was also the case with *Rhodotorula peneaus* described by PHAFF *et al.* (8). The other species of *Rhodotorula* (*R. minuta*, *R. glutinis* and *R. mucilaginis*) checked well with the descriptions given in the literature (4,5). The authors have evidence that these species are also made up of a number of physiological types.

Genus *Candida*. *Candida krusei* was the most common species encountered. It was found that the 10 strains isolated could be placed in three distinctly different groups with regard to their

carbon assimilation behavior. According to the criteria used by LODDER and KREGER-VAN RIJ (fermentation and assimilation of glucose only) they would be considered identical. In view of the discoveries by WICKERHAM and BURTON (18, 19) that many imperfect yeasts may be haploid heterothallic mating types of perfect species, we have refrained from subdividing such well known yeasts as *Candida krusei* into several additional new imperfect species, until a systematic comparative study can be made of a considerable number of strains that are now classified as a single species. WICKERHAM has shown quite clearly (19) that only strains with approximately the same assimilation scheme may be expected to belong to complementary mating types. Such detailed carbon assimilation patterns are not yet known for most imperfect yeasts. We have not attempted, therefore, to induce *C. Guilliermondii* (one isolate) to sporulate by mixing it with known mating types, even though WICKERHAM has shown that certain strains of this species constitute the imperfect form of *Endomycopsis guilliermondii* (19). Thus, all species of *Candida* were identified solely by the system of LODDER and KREGER-VAN RIJ and it was found that the isolates checked reasonably well with the descriptions given by these authors.

Genus Trichosporon. The single isolate of *Trichosporon fermentans* agreed well with the standard description of this species. Two cultures belonging to the genus *Trichosporon* could not be identified with any of the species described by LODDER and KREGER-VAN RIJ or by other workers, because of biochemical characteristics which differ from the described species. In addition they are morphologically very interesting in that numerous very sharply pointed cells, resembling needles, develop in bunches on some of the oval budding cells (see fig. 1). Because of the very characteristic needle shaped cells, the name *Trichosporon aculeatum* is proposed. One culture was isolated from *D. pinicola* near Mather (altitude 5000 ft.) and the other from *D. occidentalis* near Tioga Pass (altitude 10,000 ft.). Culture No. F-145A has been chosen as the type.

Standard description of *Trichosporon aculeatum* nov. spec.

Growth on malt extract: After 3 days at 25°C. an extensive mycelium is formed. Diameter 3.5-10.0 μ . Hyphal tips are frequently swollen. Arthrospores and small clusters of blastospores are present. The size of the blastospores is (3.5 - 4.0) \times (8.0 - 10.5). In older cultures some oval cells have outgrowths of needle-like cells, which are rounded off at the base and pointed at the other end. The needle-

like cells break off easily and many are found to be free floating in liquid preparations. The length of the needle-shaped cells is 14.0 to 23.0 μ . A heavy moist pellicle develops and eventually a mold-like growth forms throughout the flask.

Streak culture on malt agar: Color grey, surface semidull, felt-like texture, tough, strongly spreading, rather flat, with mycelial border.

Slide cultures on potato dextrose agar: Strong development of true mycelium with arthrospores in zig-zag formation. Blastospores at the septa.

Fermentation: absent (occasionally a few gas bubbles).

Carbon compounds assimilated: glucose, sucrose, maltose, cellobiose, trehalose, melezitose (latent), ethanol (weak), adonitol (latent), mannitol, sorbitol, α -methyl-D-glucoside, salicin (weak), Ca-2-ketogluconate (weak), succinic acid and citric acid.

Assimilation of nitrate: absent.

Splitting of arbutin: positive.

Production of starch-like compounds: absent.

Growth in a vitamin free medium: negative.

Acid production on chalk-agar: none.

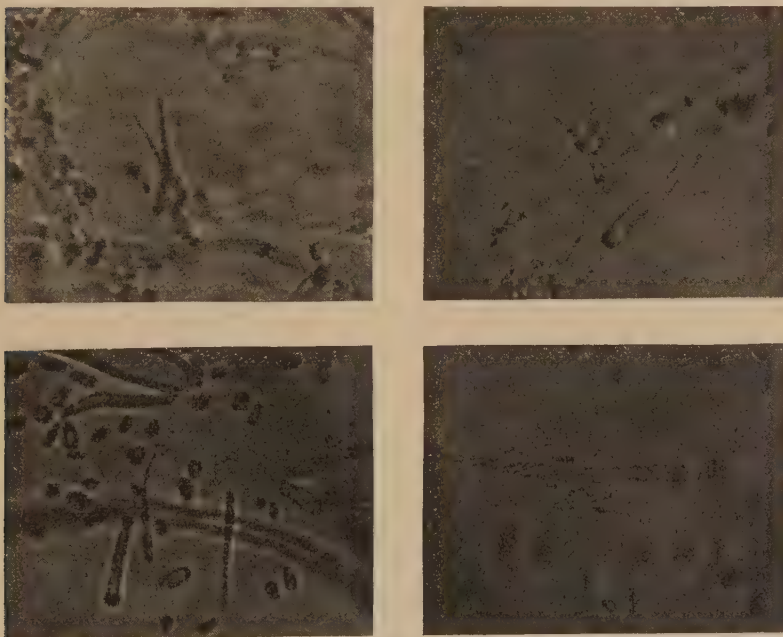


Fig. 1. *Trichosporon aculeatum* showing the typical needle shaped cells as well as true mycelium, budding cells (blastospores) and a few arthrospores.

DISCUSSION.

The yeasts which have been isolated from *Drosophila* form a

rather characteristic group in that the large majority show considerable fermentative ability. This yeast flora is quite different from the one obtained from slime fluxes and mushrooms in the same area (10) which constituted primarily an oxidative group of yeasts. It seems valid to conclude that the flies do not obtain the yeasts from these sources. Since SHEHATA and MRAK (12) showed that *Drosophila* flies digest yeast cells rapidly, the yeasts found in the crops of these insects are not permanently associated with them as parasites or commensals, but rather are ingested with their food obtained from as yet unknown sources in the area (9). Only a few systematic studies have been published on the yeasts found in *Drosophila*. Our yeasts corresponded in general with the yeast flora found by SHEHATA *et al.* (13) in flies collected primarily in Southern California, although we obtained a larger number of species. Another extensive survey was made by DUDGEON (1) who unfortunately identified her yeasts only to the genus. The isolates obtained from 21 species of *Drosophila* collected in 12 different states of the United States, (not including California) were placed in the genera *Saccharomyces*, *Hansenula*, *Pichia*, *Debaryomyces* and *Candida* (genera commonly found) and *Kloeckera* and *Torulopsis* (occurring more rarely). One significant difference compared to our survey, is the fact that *Debaryomyces* was not found by us or by SHEHATA *et al.* (13). Since species were not described by DUDGEON (1) further study will be needed to clear up this discrepancy. It would be of interest to know, for example, whether *Debaryomyces fluxorum* described by PHAFF and KNAPP (10) was present among these isolates.

LODDER and KREGER - VAN RIJ (5, pg.3) have expressed their concern that the use of a large number of carbon compounds in the assimilation tests might lead to an undesirable and impracticable splitting of existing species into dwarf species. Whereas it is probably not essential to retain all of the presently used carbon compounds for species differentiation in a particular genus, their initial use serves to indicate which compounds may be most useful for differentiating purposes after all species in a genus have been studied (16). In this connection it is quite encouraging that our results show that as many as 36 strains of a species could be isolated from nature and that only minor variations in assimilatory behavior were noted. In addition, several of the species showed essentially identical assimilation patterns when they were compared with type species obtained from the C.B.S. culture collection. Thus, increasing

the number of carbon compounds for taxonomic purposes, does not appear to lead to an uncontrolled increase in number of species.

LATIN DESCRIPTIONS OF NEW SPECIES.

Saccharomyces wickerhamii nov. spec.

In musto maltato cellulea ovoideae, $(2.1 - 5.2) \times (3.1 - 8.7) \mu$, singulae, binae aut catenatae. Sedimentum et anulus crassus formantur. In agar maltato (post unum mensem) cultura flavifusca, prope nitida, glabra, mollis, plana, margine parum undulato. Pseudomycelium nullum. Asci formatur ex transformatione cellularum vegetativarum diploidearum. Ad 4 reniformae ascosporeae in quoque asco. Glucosum, galactosum (exiguum) et saccharosum (exiguum) fermentatur at non maltosum, raffinose et lactosum. In medio minerali glucosum, galactosum, sorbosum (exiguum), saccharosum, cellobiosum, lactosum, xylosum, alcohole aethylicum (pellicula tenuis, glabra), glycerolum, sorbitolum (exiguum), salicinum, acidum lacticum, acidum succinicum (exiguum) assimilantur at non nitras kalicus. Arbutinum finditur exigua. Isolata ex *Drosophila montana* et *Drosophila pinicola*.

Saccharomyces kluyveri nov. spec.

In musto maltato cellulae ovoideae aut long ovoideae $(2.3 - 7.0) \times (2.6 - 11.5) \mu$, singulae, binae aut catenatae. Sedimentum et anulus formatur. In agar maltato (post unum mensem) cultura flavalbida, interdum crateriforma aut glabra, prope nitida, mollis, margine parum undulato, interdum parum piloso. Pseudomycelium primitivum formatur. Asci formatur ex transformatione cellularum vegetativarum diploidearum. Ad 4 ascosporeae rotundae in quoque asco. Glucosum, galactosum, saccharosum, raffinose et melibiosum fermentatur at non maltosum et lactosum. In medio minerali glucosum, galactosum, sorbosum (exiguum), maltosum, saccharosum, melibiosum, raffinose, melezitose (exiguum), alcohole aethylicum (pellicula tenuis), mannitolium, sorbitolum, alpha-methyl-D-glucosum assimilantur at non nitras kalicus. Arbutinum non finditur. Isolata ex *Drosophila pinicola* in Mather, California.

Pichia xylosa nov. spec.

In musto maltato cellulae ovoideae $(2.1 - 4.9) \times (3.5 - 7.0) \mu$, singulae, binae aut catenatae. Sedimentum, anulus et interdum insulae formantur. In agar maltato cultura flavalbida, parum nitida, interdum crateriforma, mollis, prope plana, margine parum undulato et piloso. Pseudomycelium cum blastosporis abundat. Peleiformae ascosporeae ad 4 aut 2 in quoque asco. Asci formantur ex transformatione cellularum vegetativarum diploidearum. Fermentatio exigua glucosi solius. In medio minerali glucosum, maltosum, saccharosum, cellobiosum, trehalosum, xylosum, rhamnosum (exiguum), alcohole aethylicum, glycerolum, mannitolium, sorbitolum, salicinum, acidum gluconicum, acidum lacticum, acidum succinicum, acidum citricum assimilantur at non nitras kalicus. Arbutinum finditur. Isolata ex *Drosophila miranda* in Mather, California.

Trichosporon aculeatum nov. spec.

In musto maltato cellulæ ovoideae aut cylindricae (3.5 — 4.0) \times (8.0 — 10.5) μ , mycelium verum cum arthrosporis et blastosporis. Blastosporae ovoideae aut aculeatae. Anulus et sedimentum, bene crescit post dies 21. In agaro maltato cultura griseola, non nitida, tenax, prope plana, margine piloso. Fermentatio nullum. In medio minerali glucosum, saccharosum, maltosum, cellobiosum, trehalosum, melezitiosum (exiguum), alcohole aethylicum (exiguum), adonitolum (exiguum), mannitolum, sorbitolum, alpha-methyl-D-glucosum, salicinum (exiguum), calcium-2-ketogluconicum (exiguum), acidum succinicum, acidum citricum assimilantur at non nitras kalicus. Arbutinum finditur. Isolata ex *Drosophila pinicola* et *Drosophila occidentalis* in Sierra Nevada, California.

Summary.

A survey was made of the yeasts occurring in the intestinal tract of wild species of *Drosophila* occurring in the Yosemite Region of California. Two hundred and forty one yeasts, representing 42 species and varieties, were identified. Each isolate was obtained from a different fly. Almost half of the isolates belong to *Saccharomyces*. The most common species in this genus were *S. montanus* (36 isolates), *S. veronae* (30 isolates), *S. cerevisiae* var. *tetrasporus* (22 isolates) and *S. drosophilum* (13 isolates). Further species are listed in Table 1. *Zygosaccharomyces fermentati* Naganishi was shown to be a distinct species and not a synonym of *S. cerevisiae*. In order to avoid confusion with another yeast of the same name, it has been proposed to change the name *Z. fermentati* to *S. montanus* Naganishi. Two new species of *Saccharomyces* were described, *S. wickerhamii* and *S. kluyveri*. *S. mangini* var. *tetrasporus* has been renamed *S. cerevisiae* var. *tetrasporus*. A non-cellobiose attacking strain of *S. drosophilum* has been designated tentatively *S. drosophilum* var. *acellobiosa*. A new species of the genus *Pichia* was described as *P. xylosa*. *Saccharomyces pastori* and *Saccharomyces pini* were transferred to the genus *Pichia* on the basis of arguments given in the preceding paper. A new species of *Trichosporon* was described as *Tr. aculeatum* on the basis of the presence of characteristic needle-like cells. Common species besides those mentioned in *Saccharomyces* were *Hansenula angusta* (19), *Kloeckera apiculata* (15), *Kl. magna* (13), and *Torulopsis stellata* (10). Other genera represented were *Hanseniaspora*, *Cryptococcus*, *Rhodotorula*, *Candida* and *Oospora*. Evidence was obtained that many species of imperfect genera consist of distinctly different physiological types.

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SACCHAROMYCES DELPHENSIS NOV. SPEC. A NEW YEAST FROM SOUTH AFRICAN DRIED FIGS

by

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(Received August 24, 1955).

Yeasts frequently occur in the sugary efflorescence which forms on dried figs during storage. The yeast flora associated with this sugar-like substance on Californian dried figs and prunes was studied extensively by BAKER and MRAK (1938) and MRAK and BAKER (1940). These authors examined some 230 cultures obtained from these two sources and found that almost half of their cultures were *Zygosaccharomyces* species, chiefly *Zygosaccharomyces mandschuricus* Saito and a group similar to *Zygosaccharomyces cavarae* Rodio var. *beauverie* Beauverie. The remaining cultures were representative of the genera *Torulopsis*, *Hansenula*, *Saccharomyces*, *Hanseniospora*, *Schizosaccharomyces*, *Pichia* and *Zygopichia* — in order of importance.

In a recent examination of yeasts isolated from the sugary efflorescence on a batch of dried figs from the Western Cape Province, a small-celled, ascosporegenous strain was encountered which could not be identified with any previously described species when employing the standard methods of LODDER and KREGER-VAN RIJ (1952). Characteristic features were its limited fermentative ability and the abundant formation of reniform spores (Fig. 1).

DESCRIPTION.

Growth in malt extract:

After 3 days at 25°C., the cells are oval, single or in pairs (1.5—4.0) × (2.0 — 5.5) μ (Fig. 2). After 1 month at 17°C., a sediment is formed.

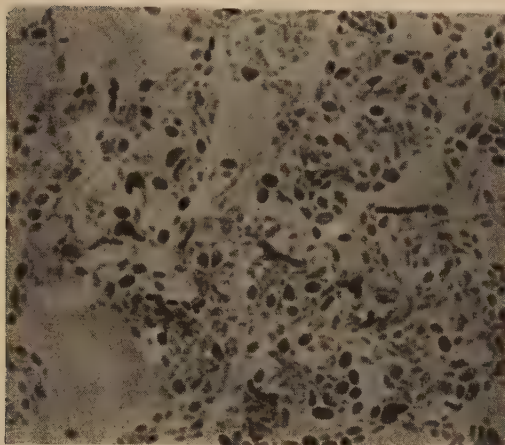


Fig. 1.

Sacch. delphensis spores on malt agar after 4 days. Schaeffer Fulton stain.
1.000 \times .

Growth on malt agar:

After 3 days at 25°C., cells have the same shape and size as in malt extract. After 1 month at 17°C., the streak culture is yellowish-brown, flat, soft, slightly raised in the middle, smooth and shining.

Slide cultures:

No pseudomycelium is formed.

Sporulation¹⁾:

Generally 4 spores are formed per ascus. Spores are reniform, but may appear long oval due to their position. They are liberated soon after formation. An isogamous conjugation usually precedes ascus formation.

Fermentation:

Glucose	+	Maltose	—
Galactose	—	Lactose	—
Saccharose	—		

Sugar assimilation:

Glucose	+	Maltose	—
Galactose	—	Lactose	—
Saccharose	—		

¹⁾ Spores were observed on malt agar and the common sporulation media.

Assimilation of potassium nitrate:

Absent.

Ethanol as sole source of carbon:

Growth.

Splitting of arbutin:

Absent.

Growth on concentrated sugar medium:

None.

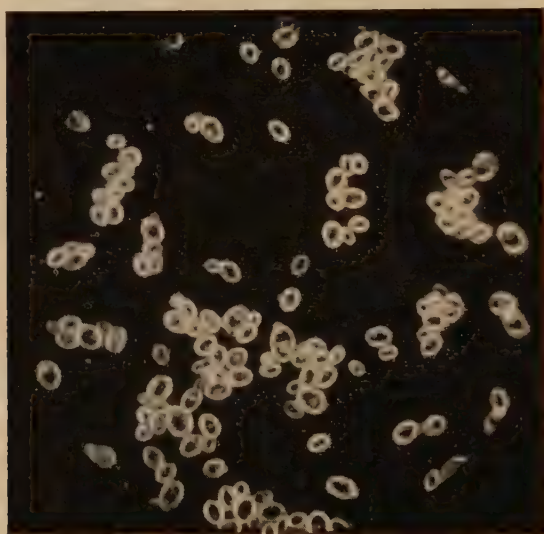


Fig. 2.

Sacch. delphensis after 3 days in malt extract. Sunlight dark-field microscopy.
1,000 \times .

DISCUSSION.

Since the organism is unable to assimilate nitrate, forms no pellicle and ferments glucose vigorously, it belongs to the genus *Saccharomyces* (Meyen) Reess as formulated by LODDER and KREGER-VAN RIJ (1952). For the species fermenting only glucose, LODDER and KREGER-VAN RIJ in the dichotomous key of the genus *Saccharomyces* employ firstly the shape of the ascospores and secondly the size of the cells for taxonomic differentiation. The formation of hat-shaped or angular spores is diagnostic for *Saccharomyces pastori*

(Guilliermond) Lodder et Kreger-van Rij 1919. Species with round to oval spores are then separated into large-celled species with cell measurements much greater than $(2.5 - 5.0) \times (3.5 - 7.0) \mu$, and species of approximately these measurements, viz.: *Saccharomyces bisporus* Naganishi (Lodder et Kreger-van Rij) 1917 and *Saccharomyces mellis* (Fabian et Quinet) Lodder et Kreger-van Rij 1928.

In the first instance, the reniform shape of the ascospores makes it impossible to classify the isolated strain as *Saccharomyces pastori*. Similarly, there is a pronounced difference from the round or oval ascospores of *Saccharomyces bisporus* and *Saccharomyces mellis*.

In the second instance, the size of the cells of the isolated strain $(1.5 - 4.0) \times (2.0 - 5.5) \mu$ is smaller than that of either *Saccharomyces bisporus* or *Saccharomyces mellis*. Its measurements indeed approximate those of *Saccharomyces pastori*. However, WICKERHAM (1955) pointed out that *Saccharomyces pastori* and the strains of *Zygosaccharomyces pini* Holst 1936, classified by LODDER and KREGER-VAN RIJ as *Saccharomyces pastori*, assimilate rhamnose, a property to which WICKERMAN (1951) attached considerable taxonomic value in his study of the genus *Hansenula*. The assimilation of rhamnose was tested for the yeast strain isolated from figs and found to be absent.

Thirdly, *Saccharomyces mellis* and *Saccharomyces bisporus* still show other minor differences from the *Saccharomyces* strain under investigation. Where *Saccharomyces mellis* is markedly osmophilic, this property is lacking in the isolated strain. Where *Saccharomyces bisporus* has never been known to form more than 2 spores per ascus, the yeast from the dried figs generally forms four.

The differences which our *Saccharomyces* strain shows from the other members of the genus seem significant enough to establish this strain as representative of a new species.

We therefore propose the name *Saccharomyces delphensis* in honour of the Delft mycologists of the Yeast Division of the Centraal Bureau voor Schimmelcultures, for their classical contributions in yeast systematics during the last quarter-century.

Saccharomyces delphensis nov. spec.

In musto maltato cellulae ovidiae $(1.5 - 4.0) \times (2.0 - 5.5) \mu$, singulae aut binae. Sedimentum formantur.

In agaro maltato formae et dimensiones cellularum eadem sunt

que in musto maltato. Cellulae copulantes et asci. Cultura (post unum mensem 17°C.) flavi-fusca, mollis, in parte media acclivis, glabra, nitida.

Pseudomycelium nullum.

Copulatio cellularum aequarum conformationi asci. Ascosporae reniformae; 4 in asco. Ascosporae ex ascis celeriter librae.

Fermentatio glucosi solius. In medio minerali cum glucosi solo crescit. In medio minerali cum alcohole aethylico crescit. Arbutinum non finditur.

The culture has been deposited in the culture collection of the Yeast Division of the Centraal Bureau voor Schimmelcultures at Delft.

A c k n o w l e d g e m e n t.

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ETUDE DE LA CROISSANCE BACTERIENNE SECONDAIRE CHEZ LES STAPHYLOCOQUES

par

E. JOIRIS

(Reçu le 28 Octobre 1955).

Les colonies de certaines espèces bactériennes peuvent, après un temps variable – et parfois dans des conditions déterminées – présenter en surface des papilles. Ce phénomène a été décrit chez les bacilles paracoli (NEISSER et MASSINI, 1906) ainsi que chez diverses entérobactériacées; nous-mêmes (FREDERICQ et JOIRIS, 1949) avons notamment étudié la production de ces papilles chez *Sh. sonnei*. Chez cette espèce, comme chez les paracoli, l'apparition des papilles est due à la multiplication de mutants capables de fermenter un hydrate de carbone que ne fermente pas la souche mère, et ne se manifeste que sur un milieu renfermant cet hydrate de carbone.

Cependant, la sélection de tels mutants ne constitue pas le seul mécanisme de production des papilles; il en est ainsi pour les papilles qui apparaissent à la surface des colonies de *N. meningitidis* (ATKIN, 1923; RAKE, 1933), *N. gonorrhoeae* (ATKIN, 1925) et les *Neisseria* du groupe "pharyngis".

Au cours du présent travail, nous avons observé et étudié le phénomène chez les souches de staphylocoques et tenté de connaître l'origine de la croissance secondaire qui constitue les papilles.

Les techniques utilisées seront décrites avec les observations rapportées dans le texte. Les souches employées ont été récemment isolées d'infections humaines dans notre laboratoire et appartenaient à l'espèce *Micrococcus pyogenes*.

OBSERVATIONS EXPERIMENTALES.

Description du phénomène.

L'observations des colonies de staphylocoques développées sur gélose nutritive ordinaire 24h. à 37°C., et laissées ensuite à la tem-

pérature de laboratoire pendant plusieurs semaines, permet de constater que la plupart d'entre elles développent des papilles à la surface (Fig. 1). Le nombre des papilles, de même que les dimensions de celles-ci, peuvent varier fortement suivant la colonie considérée. Le nombre de colonies présentant des papilles varie également suivant la souche et suivant les subcultures d'une même souche. La grande majorité des souches étudiées donnait lieu au phénomène décrit, celui-ci n'est donc pas exceptionnel.



Fig. 1. Colonies de staphylocoques après 4 semaines d'incubation à la température ordinaire. Souche S 83 (gross. $\times 25$).

Le délai d'apparition de ces papilles est variable entre certaines limites; il est de 3 à 6 semaines sur gélose ordinaire, suivant la souche ou les subcultures d'une souche donnée. L'apparition et le développement des papilles sont contemporains d'une autolyse progressive de la colonie primitive.

L'examen systématique des colonies vieillissantes, à la loupe ordinaire, ou mieux binoculaire, permet l'observation suivante, dès avant l'apparition des papilles: on peut constater, en examinant les colonies par transparence, sous un éclairage suffisant, la présence de petites colonies lenticulaires, visibles à travers la colonie primitive et localisées à l'aire occupée par celle-ci à la surface de l'agar. Si la colonie primitive est enlevée en totalité, on voit que ces petites colonies (micro-colonies) sont situées dans l'épaisseur de la gélose,

affleurant la surface de celle-ci, immédiatement sous la colonie primitive (Fig. 2).

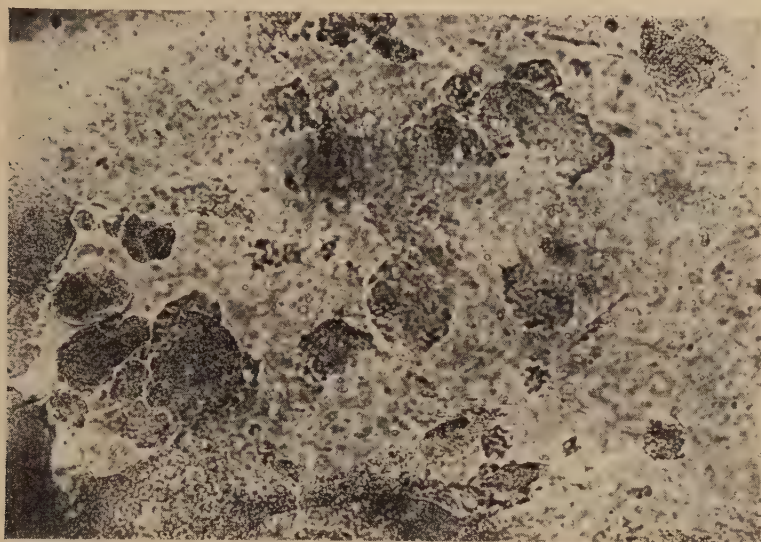


Fig. 2. Micro-colonies profondes observées après enlèvement de la colonie primitive. Incubation de 12 semaines à la température ordinaire. Souche S 649 (gross. $\times 200$).

Ces micro-colonies (M.C.) apparaissent précocement; dans certaines conditions, l'examen microscopique (gross. $\times 70$) permet de déceler leur présence après 24 h. d'incubation et, en général, dans les premiers jours du développement de la colonie primitive. Ainsi, sur gélose sang humain (10%) les M.C. sont décelables après 24 h.; sur gélose ordinaire elles le sont après 3-4 jours.

Le nombre des M.C. que l'on peut observer est très variable d'une colonie à l'autre, il est généralement compris entre 1 et 50. Leurs dimensions varient également après un temps donné d'incubation. Lorsque ces M.C. sont nombreuses, elles restent cependant localisées à une profondeur qui n'excède pas 0,5 mm, à partir de la surface de l'agar.

L'observation régulière de la croissance des M.C. montre leur développement progressif vers la surface de l'agar, sous la colonie primitive. Elles pénètrent ensuite dans cette dernière et, leur croissance se poursuivant, font saillie à la surface de la colonie primitive: une papille est alors constituée. Pendant cette évolution,

la colonie primitive s'est progressivement autolysée, son relief s'est estompé et l'opacité initiale a disparu.

Si l'observation "ab initio" permet de voir que certaines papilles observées à la surface résultent de la croissance de micro-colonies situées à l'origine dans l'agar, la dissection d'une papille bien développée permet de retrouver en profondeur le point de départ de sa croissance. Toutes les M.C. visibles au début du développement de la colonie primitive n'évoluent pas de manière identique. La plupart d'entre elles cessent de croître après un délai variable, et une minorité seulement poursuit sa croissance. Celle-ci, d'ailleurs, ne sera pas égale pour toutes les M.C. Certaines ne dépasseront pas la surface de l'agar ou de la colonie primitive et, par conséquent, ne formeront pas une papille.

La production de papilles n'est pas l'apanage exclusif de souches entretenues sur milieux artificiels pendant un certain temps. En effet, nous avons observé systématiquement sur gélose sang humain 30 cultures qui résultaient de l'isolement direct de produits pathologiques. Toutes les cultures étudiées produisaient précocement des micro-colonies et des papilles ensuite, dans les délais habituels.

Influence des conditions physiques du milieu de culture.

a. **Striation de la surface.** Lors de l'observation des micro-colonies profondes on peut constater que celles-ci sont plus nombreuses au niveau de la strie effectuée par l'öse sur l'agar, au cours de l'ensemencement. Si, après striation préalable de la surface, l'ensemencement est pratiqué par étalement sur l'agar d'une dilution convenable de la culture, le délai d'apparition des M.C. est inférieur à celui que l'on observe après étalement sans striation préalable. Une effraction mécanique de la surface de l'agar favorise par conséquent le développement des micro-colonies sans cependant être indispensable.

b. **Fluidité du milieu.** Lorsque l'isolement est effectué sur gélose semi-fluide renfermant 0,5 % d'agar, les colonies de staphylocoques présentent des contours imprécis, elles sont transparentes, fragiles, avec une zone centrale plus opaque. L'examen répété de ces colonies pendant 4 semaines ne permet pas de déceler la présence de M.C. Sur gélose renfermant 5 % d'agar, les colonies sont moins larges et plus bombées qu'en présence d' 1,5 % d'agar (concentration habituelle). Les micro-colonies apparaissent dans les délais habituels (4 à 5 jours).

Cultivées sur bouillon gélatiné à 12 %, à 18°C., les souches forment des colonies dont la croissance s'accompagne en quelques jours d'une gélatinolyse empêchant la recherche des micro-colonies.

c. **Culture sur cellophane.** Une membrane stérile de cellophane est étalée à la surface de l'agar. Après ensemencement à l'öse, on observe le développement de colonies aux contours irréguliers. Aucune micro-colonie n'est visible ni au dessus, ni en dessous de la cellophane. Cette membrane s'oppose, par conséquent, à la pénétration des bactéries dans l'agar et, ainsi, au développement des micro-colonies.

Influence de conditions reductrices.

La croissance des micro-colonies s'effectue dans un milieu où existe un certain degré de réduction. Le développement s'opère, en effet, au sein de l'agar et non en surface, directement au contact de l'air; de plus, la croissance de la colonie primitive abaisse le potentiel d'oxydo-réduction du milieu sous-jacent où se fait ce développement. Nous avons recherché si celui-ci n'était pas favorisé par les conditions réductrices plus marquées.

a. **Anaérobiose stricte.** Les cultures anaérobies sont effectuées dans la jarre de Mac Intosh en atmosphère d'hydrogène. Après 24 h. à 37°C., les jarres renfermant les cultures sont placées à la température ordinaire pendant 6 semaines, de même que les témoins cultivés à l'air libre. Dans cette atmosphère, la croissance des staphylocoques est faible et reste très inférieure à celle qu'atteignent les témoins en 24 h., malgré une incubation prolongée. Aucune M.C. n'est visible, contrairement aux témoins.

Afin de permettre un développement suffisant des colonies primitives, les cultures sont d'abord incubées à l'air libre 24 h. à 37°C. Passé ce délai elles sont placées en anaérobiose pendant 48 h. à 37°C. On peut alors observer le développement de quelques micro-colonies moins nombreuses et moins développées par rapport aux M.C. observées chez les témoins incubés à l'air libre.

Par conséquent, l'anaérobiose stricte, loin de favoriser l'apparition et le développement des M.C. exerce à cet égard un effet inhibiteur.

b. **Substances réductrices.** Diverses substances réductrices ont été incorporées à la gélose nutritive qui, après ensemencement, est incubée à l'air libre 24 h. à 37°C. puis à la température ordinaire.

Le glucose à la concentration de 1 %, le thioglycollate de sodium,

ne favorisent pas le phénomène. Le chlorhydrate de cystéine, à la concentration de 0,3 %, rend plus précoce le développement des M.C.

Potentiel d'oxydo-réduction des micro-colonies.

Le chlorure de 2, 3, 5 triphenyltétrazol (T.P.T.) est une substance qui présente la propriété d'être incolore à l'état oxydé, tandis que la forme réduite est colorée en rouge. Les cellules vivantes, dans une mesure variable suivent leur activité, réduisent le T.P.T. qui précipite alors au sein des cellules sous la forme réduite, rouge. Une solution de T.P.T., stérilisée par filtration, est ajoutée à la gélose nutritive à la concentration finale de 25 mcg.p.ml. Cette concentration permet une croissance normale de la majorité des cultures de staphylocoques.

Ce milieu a étéensemencé avec diverses souches donnant lieu au phénomène étudié. Les colonies primitives et les micro-colonies se développent dans les délais habituels. Les colonies primitives sont colorées en rouge; l'intensité et la répartition de cette coloration varient suivant les colonies. Les M.C. situées dans l'agar, sont, par contre, presque incolores. Leur développement vers la surface s'accompagnera d'une réduction progressive du T.P.T. se manifestant par l'apparition de la coloration.

Par conséquent, l'activité réductrice des micro-colonies profondes vis-à-vis du T.P.T., au début de leur développement, est inférieure à celle de la colonie primitive. Au cours de leur croissance leur pouvoir réducteur devient progressivement égal à celui de cette colonie.

Morphologie microscopique des micro-colonies.

Les bactéries constituant ces colonies, après coloration par les colorants usuels, présentent, en général, une morphologie semblable à celle des bactéries constituant les colonies primitives. Certains cocci sont plus volumineux que les cocci de l'espèce; chez d'autres, la coloration est presque exclusivement localisée à leur périphérie. Toutefois des images semblables peuvent s'observer dans les frottis de colonies normales, développées à la surface de la gélose.

Le développement initial des micro-colonies dans l'agar, au voisinage de la surface est à rapprocher du comportement des cultures du type L, dérivées des bactéries, et des "pleuro-pneumonia-like organisms". Nous avons recherché la présence éventuelle des formes L au sein des micro-colonies. Dans ce but, le bloc d'agar renfermant celles-ci est fixé dans un mélange formol-acide acé-

tique, puis écrasé entre deux lames. Les micro-colonies, fixées, restent compactes et adhèrent aux lames qui sont ensuite séparées. Après lavage et coloration, les préparations sont montées au baume et examinées au microscope. Cet examen n'a pas mis en évidence de formes L caractéristiques.

La structure nucléaire des bactéries qui constitue les micro-colonies a été observée par la technique de ROBINOW, modifiée par SMITH (1950), après écrasement du bloc d'agar renfermant les micro-colonies. Les images nucléaires obtenues ne présentent pas de différence appréciable par rapport à celles que montrent les colonies primitives. La présence de trois amas nucléaires entourés par un cytoplasme commun, décrits par SMITH chez les cultures jeunes, a été observée fréquemment.

Influence de la lyse de la colonie primitive sur l'apparition des papilles.

Le développement des papilles est contemporain d'une lyse de la colonie primitive, ainsi que nous l'avons dit précédemment. Par ailleurs, si cette lyse est accélérée par des bactéries du genre *Bacillus*, des moisissures, ou encore par la Pénicilline à concentration sub-bactériostatique, l'apparition et la croissance des papilles sont plus précoces. L'intensité de cette lyse est variable suivant la souche considérée. Nous nous sommes demandé si les produits libérés par la lyse cellulaire pouvaient être utilisés comme facteurs alimentaires par les bactéries dont la multiplication active va constituer les papilles.

Le rôle éventuel des produits de la lyse cellulaire, en tant que facteurs nutritifs, a été recherché en ensemençant les cultures sur un milieu renfermant exclusivement un autolysat staphylococcique comme élément nutritif (préparé suivant la technique de WELSCH et SALMON (1950). L'autolysat est ajouté à la concentration de 8 % à un gel d'agar renfermant 1,5 % d'agar Noble (Difco). Après ensemencement de suspensions lavées une certaine croissance s'observe sur ce milieu: après 48 h. les colonies mesurent 1 mm et conservent ces dimensions lors d'une incubation prolongée.

Les produits de la lyse cellulaire de staphylocoques peuvent, par conséquent, être utilisés comme facteurs nutritifs par cette espèce. Ainsi les bactéries constituant les papilles qui se développent sur une colonie en voie d'autolyse peuvent trouver des matériaux alimentaires dans les produits de la lyse de cette dernière.

D'autre part, les cultures en bouillon, stérilisées et solidifiées ensuite par l'agar, permettent encore un certain développement des staphylocoques, malgré leur "vaccination" antérieure, que les corps microbiens y développés soient tués par chauffage et intacts, ou qu'ils soient tués par le chloroforme et autolysés.

Propriétés de la croissance bactérienne secondaire.

Certaines papilles formées par les colonies de diverses souches ont été repiquées alors qu'elles étaient bien isolées des papilles voisines et présentaient un développement suffisant. Certaines propriétés des cultures ainsi obtenues ont été étudiées.

a. *Production de coagulase.* L'activité coagulante des cultures en bouillon de 24 h., tuées par le chloroforme, et diluées en série a été recherchée en ajoutant 0,5 ml de ces dilutions à 0,5 ml de plasma oxalaté diluée au $\frac{1}{5}$. La vitesse de la réaction et le titre final obtenu après 24 h. à 37°C. n'ont présenté que des variations peu importantes par rapport aux souches parentes.

b. *Activité hémolytique.* L'identification des hémolysines produites et la mesure de l'activité hémolytique ont été effectuées sur gélose sang de lapin, en présence d'anti-sérum suivant la technique de ELEK et LEVY (1950). Une culture dérivée d'une papille produisait une hémolysine α en concentration très supérieure à celle de la souche parente; les autres ne se distinguaient pas de la souche parente, ni par la nature, ni par la quantité des hémolysines produites.

c. *Activité antibiotique et lysogène.* Les cultures dérivées des papilles étaient dépourvues d'activité antibiotique ou lysogène sur les souches parentes.

DISCUSSION.

De ces observations, il apparaît que le développement vers la surface de micro-colonies situées à l'origine dans l'agar va provoquer l'apparition de papilles sur la colonie primitive. La formation de ces micro-colonies se réalise assez précocement, endedans les 18 h. d'incubation et paraît être due à la multiplication d'un petit nombre de cellules bactériennes, apparues au cours de la croissance de la souche, dont le métabolisme respiratoire est différent de celle-ci.

Au début de leur croissance ces variants sont capables de se développer dans une atmosphère dont la concentration en O_2 est

inférieure à celle qui existe à la surface, directement au contact de l'air, ou même exigent une telle atmosphère: ils se comportent en bactéries micro-aérophiles. Leur activité réductrice vis-à-vis du T.P.T., très inférieure à celle des individus formant les colonies en surface, indique en effet un métabolisme respiratoire différent. La majorité des individus qui constituent les populations de staphylocoques sont également capables de croître dans une atmosphère dont la tension d' O_2 est abaissée ou même nulle. Néanmoins, si les staphylocoques sont anaérobies facultatifs, ils sont nettement "aérobies préférentiels", et leur croissance est toujours plus considérable en milieu normalement oxygéné. Ce comportement les distingue des individus dont la multiplication va constituer les micro-colonies, et qui trouvent dans une atmosphère réductrice des conditions plus favorables, au moins au début de leur développement. Les générations ultérieures acquerront progressivement les propriétés métaboliques habituelles de l'espèce de sorte que la croissance s'effectuera vers la surface de l'agar, et de la colonie primitive, ensuite.

Nous avons vu qu'un milieu rendu plus réducteur, soit par suppression de l' O_2 atmosphérique, soit par incorporation de substances réductrices, n'a pas d'influence favorable sur le phénomène ou peut même inhiber celui-ci. Seule la cystéine favorise dans une certaine mesure le développement des micro-colonies. N'étant pas anaérobies stricts, mais micro-aérophiles, les bactéries en cause trouvent un degré de réduction suffisant dans le milieu environnant où s'effectue leur développement, c'est-à-dire au sein de l'agar, sous la colonie primitive.

L'effraction mécanique de la surface de l'agar favorise la pénétration dans celui-ci des bactéries qui vont constituer les micro-colonies, et rend ainsi leur apparition plus précoce. Cette effraction n'est cependant pas indispensable.

La concentration d'agar n'influence le phénomène que pour autant qu'elle soit suffisante pour permettre la constitution de colonies dans l'épaisseur du milieu.

La croissance ultérieure des micro-colonies vers la surface de la colonie primitive n'est pas due à une action antibiotique ou lysogène envers la culture parente. Elle s'effectue lorsque la colonie primitive a cessé de croître et s'autolyse progressivement. Des facteurs nutritifs, en partie dus à cette lyse, et d'autres encore présents dans le milieu de culture malgré la croissance de la colonie primitive, fournissent aux micro-colonies les matériaux nécessaires à leur croissance.

Conclusions et Résumé.

Il résulte de ces observations que les papilles apparaissant à la surface des colonies de staphylocoques, après incubation prolongée à la température ordinaire, peuvent résulter de la croissance vers la surface de micro-colonies situées à l'origine dans l'agar, immédiatement sous la colonie primitive.

Le développement de ces micro-colonies résulte de la multiplication de variants "micro-aérophiles" apparus au cours de la croissance de la colonie primitive.

Les cultures obtenues à partir de ces papilles ne se distinguent de la souche mère, ni par leur production de coagulase, ni par leur activité hémolytique, à une exception près.

La structure nucléaire des bactéries formant les micro-colonies est semblable à celle des cellules staphylococciques normales.

La recherche dans les micro-colonies d'organismes similaires aux formes L s'est montrée négative.

Summary.

After prolonged cultivation at room temperature, papillae are formed by most strains of staphylococci. This secondary growth starts as micro-colonies developing inside the agar, beneath the primary colony; these micro-colonies grow towards the surface, leading to the formation of papillae.

These micro-colonies are formed by micro-aerophilic variants developing during growth of the primary colony.

Coagulase production and hemolytic activity of cultures derived from tested papillae, are similar to that of the mother strain.

Nuclear structure of the bacteria forming the micro-colonies is similar to that of normal cells of staphylococci.

A search for L forms in the micro-colonies remained negative.

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ANTIFUNGAL PROPERTIES OF 1.2.3.4-TETRAHYDRO-9-FLUORENONE ¹⁾

by

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The antifungal properties of 1.2.3.4-tetrahydro-9-fluorenone (9) (hereafter called THF) were observed for the first time by Dr KATHERINE S. WILSON ⁴⁾ and Dr V. CUTTER (11), in a screening program of compounds synthesized in the Research Laboratories of N.V. Organon, Oss, Netherlands.

This finding instigated investigations *in vitro* into the fungistatic and fungicidal properties of this new compound, with regard to plant and human pathogens, in comparison with some other antifungal agents already known ⁵⁾.

It appeared that the spore germination inhibiting activity of THF on plant pathogens was about equal to that of CuSO₄ and much lower than that of tetramethyl-thiuram disulphide (TMTD). In contrast with this low activity, THF was found to be remarkably active when tested for growth inhibiting and fungicidal activity against dermatophytes and *Candida albicans*. In these experiments THF was compared with known drugs such as 2-dimethylamino-6- β -diethylaminoethoxy-benzothiazole dihydrochloride (DDBT) and undecylenic acid.

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On the basis of the results obtained, clinical trials with THF seem justified, provided the drug can be shown to have a low toxicity.

MATERIALS AND METHODS.

Test organisms ¹⁾.

The following plant pathogens were used as test organisms: *Alternaria tenuis* Auct., *Cladosporium fulvum* Cke., *Venturia inaequalis* (Cke.) Aderh., *Botrytis cinerea* Pers.

The dermatophytic species used were: two strains of *Microsporium gypsum* (Bodin) Guiart et Grigoraki, three strains of *Trichophyton rubrum* (Castellani) Sabouraud, and single strains of *T. schoenleini* (Lebert) Langeron et Milochevitch, *T. mentagrophytes* (Robin) Blanchard, *T. megnini* Blanchard, *M. felineum* Fox et Blaxall, *M. audouini* Gruby, *Epidermophyton floccosum* (Harz) Langeron et Milochevitch. Of the yeast *Candida albicans* (Robin) Berkhout four strains were used. The plant pathogens and *Candida albicans* were kept at room temperature, whereas the dermatophytes were incubated at 30°C.

Culture media.

The plant pathogens were grown on cherry-juice agar (*Botrytis*, *Alternaria* and *Venturia*) and on potato dextrose agar (*Cladosporium*). The dermatophytes were grown on a modified Sabouraud medium of: 4 % crude glucose, 1 % peptone, 0.1 % yeast extract "Difco", 2 % agar, in tapwater. Crude maltose (voedingssuiker, Maltostase, Weesp) was occasionally substituted for the crude glucose.

In the experiments in which the activity of THF in the presence of serum was examined, 10 % bovine serum was added to the above-mentioned modified Sabouraud medium.

Standard solutions.

The standard solutions of THF and of undecylenic acid were 1%, that of TMTD 0.1 % in ethylalcohol. DDBT was available as a 5 % solution in isopropylalcohol.

¹⁾ The test organisms were obtained from the Centraal Bureau voor Schimmelcultures at Baarn and the Yeast division of the C.B.S. at Delft.

TEST-METHODS.

A. Fungistatic.

1. **Spore germination test.** This test, applied to plant pathogens only, and used exclusively by the first mentioned author, is fully described by the Committee of Standardization of Fungicidal Tests of the American Phytopathological Society (1).

2. **The agar-plate method.** This commonly-used method was employed in most of the fungistatic experiments with dermatophytes. Graded concentrations of the antifungal compounds were incorporated into Sabouraud agar which had been molten and afterwards cooled to temperatures of 40°–50°C. The agar was immediately poured into Petri dishes of 9 cm diameter. The centre of the plate was subsequently inoculated with a small disk (5 mm diameter) punched out of a fungus mat. Plates were observed for growth once a week for about three weeks. The activity was expressed in the concentrations required for complete inhibition.

3. **The agar-cup method**, described by BURLINGAME and REDDISH (3). Spore suspensions were prepared by washing agar slants with a few ml of sterile water. Filtration of the crude suspension did not appear to be essential. Sabouraud-agar was heated and allowed to cool to 40°–45°C. The ultimate concentration in the agar was brought to about 300,000 spores per ml in the case of arthrospores of e.g. *T. mentagrophytes*, or about 3,000 spores per ml in the case of the larger and more quickly germinating macroconidia of *M. gypseum*, *M. felineum* and *E. floccosum*. 20 ml of the agar was poured into Petri dishes of 9 cm diameter in the centre of which was placed a glass ring of 1.7 cm diameter, which was removed after the agar had solidified. The plates were incubated at 30°C. As a rule after 20 hours, the cup was filled with a known quantity of 2 % water-agar, containing graded concentrations of the antifungal compound to be tested. The diameter of the inhibition zone was measured every two days in two directions, the average of the diameters being taken.

4. **The filterpaper strip method** as described by HIRSCH (7) for the evaluation of bacteriostatic agents and by BLANK (2) for the in vitro assay of antifungal compounds. Sterile filterpaper strips (85 mm long, 5 mm wide) with paperclips at one end were dipped into solutions of the compounds to be tested. The wet strips were then suspended from nails, fastened to a ledge, to dry. The dried filterpaper strips were released from the clips and placed

across the centre of the culture media. The Petri dishes were incubated at 37°C. for about 16 hours, to allow the test substance to diffuse into the medium. The next day the dishes were inoculated. A loop was dipped into the inoculum and streaks were drawn at the same height from opposite sides of the edge of the Petri dish to the strip, at right angles with the latter. The size of the inhibition zone as measured by the distance of the two corresponding fungus streaks gave the result of the assay. The inhibition zones were measured on the bottom of the Petri dish.

5. The filterpaper disk method. Agar plates containing spore suspensions were prepared as described for the agar cup method. Five filterpaper disks (Carl Schleicher and Schuell no. 2247, diameter 1.25 cm) impregnated with various concentrations of THF, were placed all on one plate. The molar concentrations amounted to resp. 0.02 – 0.01 – 0.005 – 0.0025 – 0.00125 m. Beginning in the centre of the disks the radius of the inhibition zone was measured in two directions on the bottom of the Petri dish. Here again the average of the radii was taken. Radius of the disks 6.25 mm.

B. Fungicidal.

Our method was based on that of BURLINGAME and REDDISH (3) as modified by GOLDEN and OSTER (4 and 5) and by WALKER *et al.* (10). Culture mats were exposed to various concentrations of the fungicide. At fixed times (1, 5, 15 and 30 min.) previously punched disks were taken out of the plate. Then the fungicide was removed from the disks by washing in 10 ml of 25 % aqueous ethanol for fifteen minutes, and the ethanol in its turn by washing in 10 ml of sterile water for another fifteen minutes.

Subsequently the disks were brought on Sabouraud slants and examined for growth after fourteen days. Since the concentrations tested in this way are always very high (1000 – 4000 p.p.m.) with a view to killing off the fungus in as short a time as possible, we looked for a modification which would enable us to test the minimum lethal dose for a given compound during a certain more prolonged time. As ethanol could not be used as a diluent in this case because of its own antifungal activity, we turned to the agarplate method, incorporating into the agar higher concentrations than the lowest fungistatic ones already found in earlier experiments.

18–20 Mycelial disks (5 mm diameter) were placed on the plates,

culture side down, and incubated at 30°C. After 1, 3, 5 and 10 days resp., some of the disks were washed in ethanol and water, and put on Sabouraud slants as described above.

DISCUSSION OF THE VARIOUS METHODS.

The agar cup, the filterpaper strip and the filterpaper disk methods are all based on the principle of diffusion from a centre where the concentration is very high.

Consequently the inhibition zone, formed after some time, may fail to demonstrate that the concentration in the whole plate has become too high for the mycelium in the border ring to grow any further. One could as well conclude that in this inhibition zone every germ was killed before the concentration in the plate was evened out.

With THF this was demonstrated by punching disks out of the inhibition zone, at different distances from the centre, and treating them as described for the evaluation of fungicidal activity. Only the germs nearest to the ring of growing mycelium appeared not to have been killed.

A similar result was observed when the cup had been filled after an incubation time of one or two days. However, when THF was brought into the cup after three days, it was no longer lethal to the germs in the concentrations used, but only prevented the growth in the zone around the cup. Another disadvantage of these diffusion methods is that the inhibition zone will steadily decrease if the fungus or the medium is able to inactivate the antifungal compound. On the other hand it should be kept in mind that the fungus will always grow centripetally if the resulting concentration of the compound in the whole plate, including the cup, is lower than the minimum fungistatic dose. The agar plate method would entirely mask the effect of inactivation, which is a still greater disadvantage. Only the fact that the lowest fungicidal dose appears to be just a little higher than the lowest absolutely fungistatic one, might be an indication for this phenomenon.

It may therefore be concluded that neither the agar plate, nor the agar cup method (or some other method based on the diffusion principle) should be employed exclusively if the fungistatic activity of an unknown compound is to be evaluated.

The agar cup procedure does not allow the lowest fungistatic

dose to be determined exactly. Procedures based on the diffusion principle are valuable only for purposes of comparison: they give exact information neither on the diffusion rate of the compound, nor on the fungistatic activity.

Besides it is conceivable that the compounds inducing equal inhibition at equal concentrations in agar, might not have the same diffusion rate when diffusing in skin, nails, hair or other tissues. Therefore, while some valuable suggestions in the course of these investigations were derived from diffusion methods, reliable results, both for the fungistatic and for the fungicidal activity, were obtained by the agar-plate method only.

DISCUSSION OF RESULTS.

A. Fungistatic properties.

a. Fungistatic activity of ethanol.

As the drugs tested were dissolved in ethanol, it appeared essential to know how much of this solvent had any noticeable antifungal activity. It was found that concentrations of only 0.25–0.5 % were slightly fungistatic to *T. rubrum* and *M. gypseum* as were concentrations of 1–2 % to *C. albicans*. As the resp. lowest fungistatic doses of THF against these organisms correspond to 0.2% ethanol in the first case and to 1% in the last one, the influence of ethanol may be neglected.

b. Plant pathogens.

Slide germination tests.

As is shown in table 1, which gives LD 50 of the compounds tested, THF is hardly more powerful than CuSO_4 and much less powerful than TMTD.

TABLE 1.

LD 50 of THF, CuSO_4 and TMTD obtained according to the slide germination test, in p.p.m.

	THF	CuSO_4	TMTD
<i>Botrytis cinerea</i>	14	14	0.4
<i>Alternaria tenuis</i>	4	8	1.4
<i>Alternaria brassicae</i>	3.5	2	0.8
<i>Venturia inaequalis</i>	3	5	0.03
<i>Cladosporium fulvum</i>	3.5	6	0.35

Agar cup method.

The results obtained in experiments performed with the agar cup method appear to be in accordance with these findings, as is shown for *Botrytis cinerea* in table 2.

In view of these results there is no reason to recommend THF for the control of fungus plant diseases.

TABLE 2.

Antifungal activity of THF, CuSO_4 and TMTD against *Botrytis cinerea* as obtained with the agar cup method. Zone of inhibition in cm.

p.p.m.	4000	2000	1000	500	250	125	62.5	31.2	0
THF	—	—	3.5	3.2	3.0	2.4	1.9	1.7	1.7
CuSO_4	2.7	2.2	1.7	1.7	1.7	1.7	1.7	1.7	1.7
TMTD	—	—	—	6.0	5.2	5.1	5.0	4.1	1.7

c. Human pathogens.

Here again THF was compared with known antifungal compounds.

GRUNBERG *et al.* (6) showed that in plate tests DDBT inhibits the growth of dermatophytes in dilutions as low as 1 – 2,000,000.

Table 3 shows that as a rule the lowest fungistatic dose of THF is lower than that of DDBT. In both cases the dose varies considerably with the fungus species tested, but more so in the case of DDBT than in that of THF. It was noted that DDBT is more active in slowly growing species such as *T. megnini* and *M. audouini* than in quickly growing ones as *M. gypseum* and *T. rubrum* str. Cast.; for THF no such differentiation could be found.

TABLE 3.

Lowest fungistatic dose of THF and DDBT in p.p.m., as determined with the agar plate method, after a 20 days' incubation. The diameter of the controls at this moment is given in order to show the difference in growth rate.

organism	THF	DDBT	diameter of the control in cm
<i>M. gypseum</i>	26	160	8.9
<i>T. rubrum</i> str. Castellani	13	60	8.4
<i>T. rubrum</i> str. Grosfeld Doubs	40	160	6.3
<i>T. rubrum</i> str. Rivalier	23	50	4.5
<i>M. audouini</i>	20	40	5.3
<i>T. megnini</i>	30	10	5.6

Table 4 gives the results of agar cup experiments with THF, DDBT and undecylenic acid. Apparently undecylenic acid, much used clinically, is far less active *in vitro* than either THF or DDBT.

TABLE 4.

Fungistatic activity of THF, DDBT and undecylenic acid against *M. gypseum*. Zone of inhibition in cm.

Concentration in p.p.m.	THF	DDBT	Undecylenic acid
625	6.2	4.8	2.1
156	6.1	3.9	1.7
39	4.7	2.8	1.7

A difference in activity of THF and DDBT worth mentioning is shown in fig. 1, in which the diameter of the quickly growing *T. rubrum* str. Cast. is plotted against the concentration of the drug. In the higher concentrations THF is much more effective than DDBT, whereas in the lower doses DDBT appears to be more active.

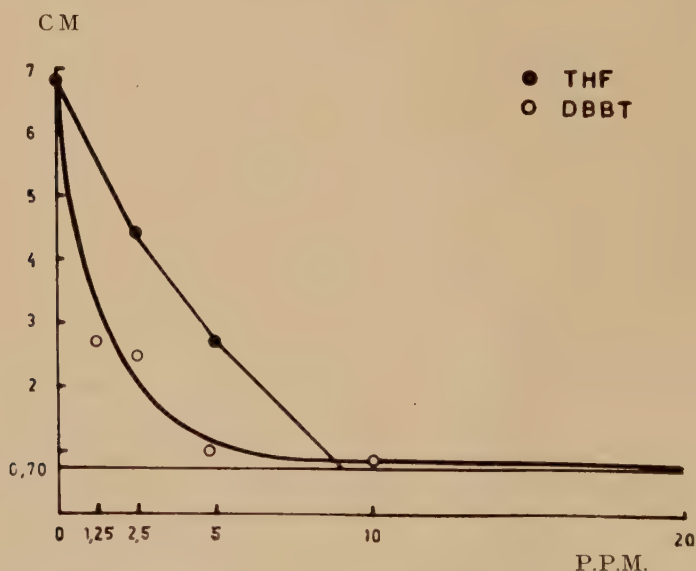


Fig. 1. Influence of THF and DDBT on the growth of *Trichophyton rubrum* str. Castellani. Duration of the experiment: 16 days. Original diameter of the mycelial disks: 0.70 cm.

d. THF and serum.

The above experiments were made without the addition of serum to the medium. As *in vitro* some substances showing fungistatic activity are partly or wholly inactivated in the presence of body fluids or animal tissues, a series of experiments was carried out in order to investigate the influence of serum.

Unlike BLANK (2) we could hardly detect any noticeable growth promoting activity of bovine serum when added to the Sabouraud medium in percentages of 1 up to 10. However, it markedly decreased the activity of THF.

TABLE 5.

Minimum inhibitory THF concentration in p.p.m. determined by the agar plate method after a 20 days' incubation.

organism	no serum	serum 10 %
<i>T. rubrum</i> st. Cast.	10	80
<i>M. gypseum</i>	30	160
<i>E. floccosum</i>	40	100
<i>C. albicans</i>	175	400

TABLE 6.

Effect of various molar THF concentrations on the growth of *T. megnini* and *T. schoenleini* as measured by the filterpaper strip method.

organism	ns	s	ns	s	ns	s	ns	s	ns	s
	0.125 m		0.25 m		0.5 m		1 m		2 m	
<i>T. megnini</i>	0	0	4.6	0	6.1	1.5	7.0	3.0	—	6.1
<i>T. schoenleini</i>	0	0	2.3	0	4.0	0	6.5	3.1	—	5.0

The distance between two corresponding fungus streaks is given in cm. ns = plain medium, s = medium + serum; — = no growth at all.

TABLE 6a.

Effect of various molar THF concentrations on the growth of two strains of *C. albicans*. Filterpaper strip method. Same designations as table 6.

strain	ns	s	ns	s	ns	s	ns	s
	0.04 m		0.02 m		0.01 m		0.005 m	
smooth	4.5	1.8	3.7	1.1	0.7	0	0.8	0
rough	4.9	2.7	3.8	1.5	1.2	0	0.8	0

TABLE 7.

Effect of various molar THF concentrations on the growth of *M. gypsum* as measured by the filterpaper disk method.

ns	s	ns	s	ns	s	ns	s	ns	s
0.02 m.		0.01 m.		0.005 m.		0.0025 m.		0.00125 m.	
A —	2.0	—	1.1	—	0.8	1.4	0	0.9	0
B —	1.9	—	1.3	1.9	1.0	1.3	0	1.2	0

Radius of total inhibition zone after a 7 days' incubation is given in cm; A = disks adjusted at once; B = disks adjusted after a 20 hours' incubation at 30°C; ns = plain medium; s = medium + serum; — = radius more than 3.0 cm.

From *T. megnini* and *T. schoenleini* mycelial suspensions were made, as contrasted to *M. gypsum* and *C. albicans* from which spore suspensions were used. This difference, rather than other variations in test method may most probably account for the big differences between the fungistatic doses in tables 6 and 6a.

At the end of the experiments summarized in tables 5, 6 and 7, disks were cut out of plates in which the THF concentrations appeared to be fungistatic, washed and put on plain Sabouraud agar. In all cases the platings showed definite signs of growth.

From tables 5, 6 and 7 we can conclude that, under the conditions of the test, in the presence of bovine serum the potency of THF against dermatophytes is reduced to more than $\frac{1}{4}$ of its value in the absence of serum.

In *C. albicans* the reduction is from $\frac{1}{2}$ to $\frac{1}{3}$, a result which is obtained also from experiments using the filter strip method (See table 8). One possible explanation of this may be degradation of the serum by the yeast.

According to SEALE (9) the activity of DDBT is reduced to $\frac{1}{8}$ of its original value in the presence of serum, when tested under similar conditions.

B. Fungicidal properties.

When the fungicidal activity was evaluated according to the usual method, the results given in table 8 were obtained.

DDBT was not lethal to *C. albicans* in these experiments.

For reasons mentioned above further experiments were carried out with a modification of this method, the organism being brought

TABLE 8.

Fungicidal activity of THF and DDBT. Contact periods necessary for lethal effect of 2000 p.p.m.

Organism	Contact period (minutes)	
	THF	DDBT
<i>C. albicans</i>	16	—
<i>T. rubrum</i> str. Cast.	10	15
<i>M. audouini</i>	5	15

TABLE 9.

Fungicidal activity of THF with 10 % serum added to the medium.

organism	Contact period (days)							
	THF, 160 p.p.m.				THF, 320 p.p.m.			
	1	3	5	10	1	3	5	10
<i>M. gypseum</i>	+	+	+	+	+	±	—	—
<i>T. megnini</i>	+	±	±	—	+	—	—	—
<i>T. rubrum</i>	+	—	—	—	—	—	—	—
<i>M. audouini</i>	+	+	+	+	—	—	—	—

+ = growth, ± = growth of some of the disks,
— = no growth.

TABLE 10.

Fungicidal activity of THF and DDBT against *M. gypseum*.

antifungal agent		conc. in p.p.m.	contact period (days)			
			1	3	5	10
THF	ns	40	±	—	—	—
		80	—	—	—	—
THF	s	160	+	—	—	—
		320	—	—	—	—
DDBT	ns	160	+	+	+	±
		320	+	+	±	±
		640	+	+	±	—

into contact with lower doses during longer periods of time. The results obtained (tables 9 and 10) are more detailed and accurate than those given in table 8.

The fungicidal activity of THF appears to be higher than that of DDBT. As is also shown in fig. 1, THF entirely suppresses growth at concentrations where DDBT only inhibits it.

As mentioned above, when the agar-cup method was used for testing THF and DDBT, the fungus in the inhibition zone appeared to be killed when THF was in the cup. When DDBT was tested, however, growth was observed of the fungus, correctly washed and placed in a Sabouraud slant as explained above, even in disks punched from the immediate vicinity of the cup.

The fact that the antifungal activity of THF appears to be more of a fungicidal than of a fungistatic nature may possibly be of importance with respect to its clinical usefulness in the treatment of mycoses.

S u m m a r y.

1. Investigations are described of the fungistatic and fungicidal activities of a new antifungal compound, 1.2.3.4.-tetrahydro-9-fluorenone (THF).
2. The spore germination inhibiting activity against plant pathogens is about equal to that of CuSO_4 and much lower than that of tetramethylthiuram-disulphide.
3. Against dermatophytes and *Candida albicans* THF has been found to be a very potent agent.
4. The antifungal activity of THF appears to be more of a fungicidal than of a fungistatic nature, whereas for 2-dimethylamino 6- β -diethylaminoethoxy-benzothiazole dihydrochloride (DDBT) the reverse seems to be the case.
5. The presence of serum diminishes the activity of THF on dermatophytes for about four times.
6. Described is a modification of the agar plate fungistatic test method which is applied for fungicidal testing.

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SACCHAROMYCES TRANSVAALENSIS NOV. SPEC. A NEW YEAST FROM SOIL

by

J. P. VAN DER WALT

(Received November 14, 1955).

Although the function and activity of the yeasts in soil are not fully understood, these organisms appear to be present in most surface soils. In a recent survey of South African soils a large-celled, sporogenous species was isolated from this source. Classification according to the standard procedures of LODDER and KREGER-VAN RIJ (1952) showed this species to be novel.

Characteristic of the strain was its limited fermentative ability, fermenting only glucose and galactose, and the formation of one and occasionally two large, round to oval, spores per ascus.

DESCRIPTION.

Growth in malt extract: After 3 days at 25 °C. two

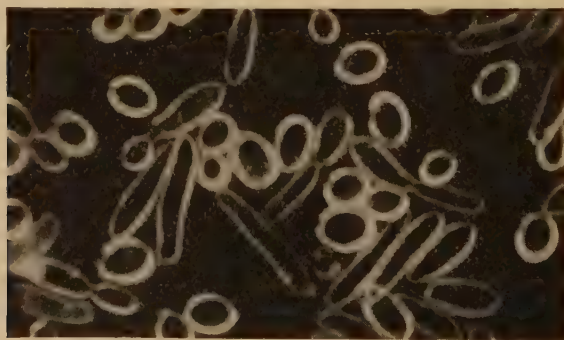


Fig. 1. *Sacch. transvaalensis*. After 3 days in malt extract (1000 \times , sunlight dark field microscopy).

types of cells are formed, oval to long oval or greatly elongated $(3.6 - 7.2) \times (4.7 - 11.4 - 25) \mu$, single or in pairs (Fig. 1). After 1 month at 17 °C. a sediment, ring and a few islets are formed.

Growth on malt agar: After 3 days at 25 °C. the cells are either oval to long oval or greatly elongated $(3.1 - 7.8) \times (4.2 - 12.5 - 37) \mu$, single or in pairs. After 1 month the culture is cream-grey, glistening of slightly dull, smooth or wrinkled with little warts.

Slide culture: A primitive pseudomycelium is formed.

Sporulation¹⁾: The spores are large, round to oval, 1-2 per ascus, mostly 1. (Fig. 2).

Fermentation:	Glucose	+	Maltose	—
	Galactose	+	Lactose	—
	Saccharose	—		

Sugar assimilation:	Glucose	+	Maltose	—
	Galactose	+	Lactose	—
	Saccharose	—		

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: No growth.

Splitting of arbutin: Absent.

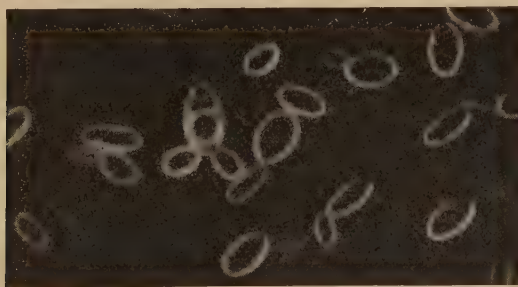


Fig. 2. *Sacch. transvaalensis*. Spores on Gorodkova agar after 6 days (1000 \times , sunlight dark field microscopy).

DISCUSSION.

The absence of pellicle formation, the inability to assimilate nitrate and the vigorous fermentation of glucose define the strain as a species of the genus *Saccharomyces* (Meyen) Reess as formulated by LODDER and KREGER-VAN RIJ.

¹⁾ Spores were observed on malt agar and on the common sporulation media.

Biochemically the strain resembles *Saccharomyces delbrückii* Lindner and its variety *mongolicus*, which are the only members of the genus fermenting glucose and galactose. Morphologically, however, it is quite distinct. Whereas the mature, round to short oval cells of *Sacch. delbrückii* and the short oval to oval cells of the variety *mongolicus* measure $(2-5) \times (2.5-6.5) \mu$ and $(3.5-6) \times (5-10) \mu$ respectively in malt extract, those of the strain from soil measure $(3.6-7.2) \times (4.7-11.4-26) \mu$ under the same circumstances. A minor morphological difference may still be seen in that the strain from soil forms a primitive pseudomycelium, which property is lacking in *Sacch. delbrückii* and in its variety.

The strain must therefore be regarded as representative of a new species. The name *Saccharomyces transvaalensis* is proposed, naming it for the province from whose soil it was isolated.

The culture has been deposited in the Yeast Collection of the Centraal Bureau voor Schimmelcultures in Delft.

Saccharomyces transvaalensis nov. spec.

In musto maltato cellulae aut ovidiae longovidiaeque aut productae multae $(3.6-7.2) \times (4.7-11.4-26) \mu$ singulae aut binae. Sedimentum, parvae insulae et annulus formantur.

In agar maltato cellulae aut ovidiae longovidiaeque aut productae multae $(3.1-7.8) \times (4.2-12.5-37) \mu$ singulae aut binae.

Cultura (post unum mensem, 17°C.) griseola albida, parum nitens, glabra, in parte rugosa cum verrucae parvae.

Pseudomycelium primitivum formatur. Ascosporae magnae, rotundae aut ovidiae; 1-2 in asco, plerumque 1.

Fermentatio glucosi et galactosi. In medio minerali cum glucoso et galactoso crescit. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico non crescit. Arbutinum non finditur.

A c k n o w l e d g e m e n t.

The author's thanks are due to Professor A. PIJPER for his kind assistance with the photography.

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OBSERVATIONS ON THE LYOPHILIZATION OF *BRUCELLA ABORTUS*, STRAIN 19

I. IMPROVEMENT OF THE SURVIVAL RATE

by

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(Received August 15, 1955).

INTRODUCTION.

Since 1951 the State Serum Institute has delivered the Str. 19 vaccine against bovine brucellosis in the lyophilized form only. Initially more than 50% of the viable cells, present in the original suspension perished during the freeze-drying process. As a result of this, along with the prescribed number of 60×10^9 viable cells of *Brucella abortus* Str. 19, each dose contained a considerable ballast of dead cells. In view of qualitative considerations (VAN DER SCHEER and EISMA, 1954) a reduction of this dead ballast was judged to be necessary. This implicated the task to increase the survival rate of the freeze-drying process as much as possible.

By survival rate is meant the percentage of viable cells of the original suspension still present immediately after drying.

METHODS.

The investigation had to be based entirely on viable counts of *Brucella abortus* Str. 19.

In bacteriology usually it is considered to be sufficient to estimate the approximate viable count in the material studied. Hence the accuracy of counting methods usually does not meet severe standards.

For the present investigation, however, it was essential to have the disposal of an accurate counting method. A satisfactory accuracy was arrived at with the following method:

Dilutions are made by distributing each time 0.1 ml in 100 ml diluent, as far as this is possible. Thus the number of steps by which the final dilution is reached, is reduced in order to limit the possible error. The volumes of diluent are measured accurately, or even remeasured after the dilutions have been made.

In order to arrive at a sufficient accuracy in pipetting 0.1 ml-amounts, essayed capillary pipettes with a long-drawn calibrated scale are used. The outside of the pipettes has to be dried carefully before adjustment to zero. For this the sterile inside of a cottonplug of a discarded test tube may be used.

From the final dilution 10 separate streaks or droplets of 0.01 ml each are brought on a previously dried tryptose-agar plate. The inoculum is not distributed any further. Before being absorbed into the agar it spreads sufficiently over the surface of the plate to produce well countable, mostly discrete colonies. The number of viable cells per inoculum of 0.01 ml should lie between 10 and 40.

Inhibiting substances (such as gentian violet) which may invalidate the regularity of the counts must not be added to the medium.

The colonies are preferably counted after 3 days incubation at 37°C. After a longer incubation neighbouring colonies will coalesce thus bringing down the counts. This error will be comparatively higher in samples with greater numbers of viable cells per inoculum.

TABLE I.

Viable counts $\times 10^9$ in the dried final product in a series of 15 ampoules from one batch.

	Count per ampoule (2 plates)	Viable count (mean of 3 ampoules = 6 plates)	Total mean (of 15 ampoules = 30 plates)
I	538	570	593
	573		
	598		
II	622	606	
	607		
	590		
III	635	588	
	584		
	545		
IV	636	606	
	588		
	593		
V	582	597	
	621		
	587		

All viable counts were performed in triplo *viz.*, always 3 separate samples were diluted and from each final dilution two plates were inoculated. A single viable count was thus obtained from the counts of 6 plates.

Table 1 gives an impression of the degree of accuracy attained by the above methods.

From these data may be calculated that the standard deviation of the count per ampoule is about 5% of the mean and for a viable cell count (counts of 3 ampoules = 6 plates) nearly 3% of the mean. As the above counts have been carried out after drying, the sampling error in the filling of the ampoules with an automatic pipette, as well as any deviations in the survival rates, due to differences in the placing of the ampoules during the desiccation or to differences in their final sealing are included in the standard deviation just mentioned.

For the greater part the same conditions prevailed during the test dryings, the only difference being that the ampoules under test were always put in fixed places, arranged radially in the circular drying racks, the sets of controls and other ampoules to be compared neighbouring them as near as possible. If this difference had any influence it may only have enhanced the accuracy of the counting method. As furthermore all countings were carried out by the same analyst, it will be allowed to reckon with a standard deviation of 3% of the mean for all viable counts during this investigation.

It is interesting to compare these data with those presented by HUDSON, HULSE and COLLINS (1954) over a large series of counts from nearly analogous, dried test material. From these data can be computed roughly that the standard deviation of a count per ampoule surpassed 20% of the mean which compares with the standard deviation of 5% of the mean per ampoule in our series.

The method for culturing *Brucella abortus* Str. 19 and the lyophilization procedure in modified Edward's dryers with separate freezing have already been described in an earlier paper from this institute (BOSGRA 1951).

Meanwhile at the start of this investigation, the following modifications in these methods had been introduced: The serum was left out of the medium, after this was shown not to influence the yield. The suspending medium, originally composed of a 7% glucose solution was replaced by a 2% tryptose, 0.5% neutralized ascorbic

acid and 0.25% thiourea solution which allowed a more effective preservation of the dried vaccine. Eventually a permanent apparatus of larger capacity was substituted for the provisional device for separate freezing of the ampoules.

FACTORS WHICH MAY INFLUENCE THE SURVIVAL RATE.

For a certain bacterium the survival rate after lyophilization will depend on the methods used and the composition of the suspending medium.

Losses during freezing.

The first losses of viable cells may occur already during freezing of the suspension. According to FLOSDORF (1949) at first it was generally accepted that temperatures of -70°C . or below had to be applied in the freezing process. From the investigations of WEISER and OSTERUD (1945) and of HUTTON, HILMOE and ROBERTS (1951) it appeared however, that especially at 0° to minus 20°C . considerable losses of viable cells occur. At lower temperatures these losses diminish rapidly.

Hence in freezing of the bacterial suspension the interval between 0° to -20°C . has to be passed as quickly as possible. Once a lower temperature is reached, it may be accepted that any further loss of viable cells will be slight. Since the new apparatus for freezing of the suspensions has been put into use, these requirements for adequate freezing are sufficiently met.

Losses during drying.

According to HUTTON, HILMOE en ROBERTS (1951) also during the drying process a temperature below -20°C . would be favorable for obtaining a high and constant survival rate for *Brucella abortus*. According to others (PROOM, 1951; STABLEFORTH, 1951) quite satisfactory survival rates might be obtained at higher temperatures as well. So literature is not unanimous on this subject.

Until a generally accepted conclusion has been arrived at it will be safe to limit a possible rise in temperature during the drying process. With the Edward's dryers used, this condition can be easily met.

Reduction of the losses by additions to the suspending medium.

At first the only additions to the suspending medium known to increase the survival rate in the lyophilization of bacteria were

“protective” proteins. FLOSDORF (1949) mentions serum, plasma and skim-milk as examples of such media. PROOM (1951) further mentions broth while also the addition of gelatin or methylcellulose would reduce the loss of viable cells during freezing. He considers the occurrence of large molecules which might act as protective colloids as the only property these media have in common. LESHCHINSKAYA (1944, cited by FRY) obtained good results by drying B.C.G.-vaccine in glucose. FRY (1951) confirmed that several sugars in concentrations between 5 and 10% could markedly increase the survival rate in the drying process of various bacterial species, the more so when protective colloids were also present.

STAMP (1947) discovered that ascorbic acid, either neutralized or not, would act favorably in the drying of bacteria.

During drying experiments with *Serratia marcescens*, NAYLOR and SMITH (1946) noted that also other reducing substances such as thiourea (thiocarbamide) increased the survival rate and might partially be substituted for ascorbic acid. Various investigators (VERWEY and SCHEIDY, 1946; BOSGRA, 1951 and THORNE, 1953) added different combinations of protecting substances to the suspending media for the production of lyophilized Str. 19 vaccine. Despite these differences in the suspending media, consistently survival rates of approximately 50% were obtained. Indeed the composition of the drying media concurred in so far, that apart from sugars reducing substances were never included. Hence it was striking that in a few experiments with *Brucella abortus* (BOSGRA and VAN KLINKENBERG, 1947; HUTTON, HILMOE and ROBERTS, 1951) where reducing agents had been added to the medium incidental survival rates of about 70% had been reached. Apparently this pointed to the possibility that the addition of reducing substances might increase the survival rate of *Brucella abortus* considerably above 50%.

Contrary to this indication BOSGRA (1951) in the large-scale production of lyophilized abortus vaccine did not succeed in reproducing a reasonable survival rate when using the medium of BOSGRA and VAN KLINKENBERG (1947) which contained reducing substances. With a solution of glucose as suspending medium he obtained better results.

As has been mentioned above, later on a suspending medium containing tryptose, ascorbic acid and thiourea was substituted for the glucose solution in view of a more effective preservation of

the final product. Notwithstanding the presence of reducing substances in this new medium, the average survival rate calculated over a large number of batches here also remained below 50%. These experiences hardly supported the former indications that the survival rate of *Brucella abortus* might be brought up to about 70% by adding reducing substances to the suspending medium.

When, however, reducing agents are added to a suspending medium, the assumption is self-evident that their beneficial action is due to their reducing power. So this reducing capacity ought to be present in the suspending medium as completely as possible (high redox potential) during freezing and drying.

Now in the procedure originally followed the reducing substances were already present in the suspending medium with which the Str. 19 cultures were washed from the agar. During this manipulation the suspending medium was stirred continuously thus being exposed to the air in a thin layer and at temperatures of 35—25°C. The presence of glass-beads still increased the exposed surface considerably. Finally the suspension was kept at 4°C. for 2 or 3 days for the checking of purity before being dried. Taking into account that substances such as ascorbic acid are oxydized very easily it could be taken for granted that the reducing capacity of the suspending medium would have decreased substantially and might even have completely disappeared (low redox potential).

OWN INVESTIGATIONS.

In several batches of Str. 19 suspension, harvested in the way described, and filled into ampoules, a certain extra amount of freshly prepared suspending medium was added to a number of ampoules immediately before freezing and drying. After lyophilization the viable count in the ampoules with the extra addition was always found to be higher than in those without.

In view of this result in another series of test-ampoules the reducing substances were completely left out of the suspending medium, but equivalent amounts were added as a fresh concentrated solution, to the Str. 19 suspension immediately before lyophilization (as suggested already by STAMP). The suspending medium in which the Str. 19 cultures were harvested and kept for the purity control, therefore did not contain but a 2% tryptose solution. After having estimated the increase in the survival rate to be expected when

putting this modification into practice, the new method was introduced in the vaccine production.

Calculated over the next 14 batches, the survival rate, which had fluctuated around 56% for the last months, now rose to an average of about 73%. This result was reached in a suspending medium of quantitatively exactly the same composition as the medium in which the survival rate of 56% had been obtained, *viz.*, 2% tryptose, 0.5% neutralized ascorbic acid and 0.25% thiourea. Therefore the difference in both media was qualitative and the different survival rate must have been the result of a difference in redox potential.

Under the modified conditions it might be possible that also quantitative changes in the suspending medium would induce still further improvement of the survival rate.

In a detailed investigation NAYLOR and SMITH (1946) found that in the lyophilization of *Serratia marcescens* an optimum survival rate was obtained when next to 0.5% ascorbic acid, 0.5% thiourea was added. The addition of 0.5% NH_4Cl would increase the survival rate still further. Their medium contained as a protective colloid 2% dextrine. HUTTON, HILMOE and ROBERTS (1951) applied about the same medium in the drying of *Brucella abortus*. They added already 0.1% tryptose and besides 0.25% NaCl. They did not, however, investigate whether the ratios recommended by NAYLOR and SMITH for *Serratia marcescens* were the most favorable for the lyophilization of *Brucella abortus* as well.

Therefore a further investigation was undertaken into the influence of changes in the concentrations of ascorbic acid and thiourea and the addition of NH_4Cl , NaCl and dextrine to the suspending medium on the survival rate of *Brucella abortus*. For this investigation the usual medium, which in the meantime had proved to give favorable results was used as a starting point. To this basic medium the various substances mentioned were added in increasing amounts.

Eventually the influence of an addition of glycerol and glucose was studied. In these two series, however, the content of thiourea in the basic medium was 0.75% instead of 0.25%.

To estimate the value of the experimental results obtained, the probable error in the viable counts has to be taken into account. In discussing the methods of investigation it was already concluded,

that for the viable counts a standard deviation of 3% of the mean has to be reckoned with.

When comparing two viable counts the standard deviation to be allowed for the difference between the counts will be $\sqrt{2} \times$ as high, while to obtain a 95% reliable interval a further multiplication with 2 is necessary. This means that the difference between 2 viable counts is real, with a reliability of 95% when this difference exceeds $3 \times \sqrt{2} \times 2 = 8\frac{1}{2}\%$.

In the tables 2 and 3 the viable counts after drying in the basic medium are always put at 100, whilst the viable counts determined after drying with the various additions are calculated as percentages of the former. Whenever the difference between the counts in basic medium and medium with additions exceeds $8\frac{1}{2}\%$ this is considered to indicate a real difference and the values are printed in bold type.

TABLE 2.

Basic medium: 2% tryptose, 0.5% neutralized ascorbic acid and 0.25% thiourea.

Increase of the ascorbic acid content up to:

0.5% = 100

Conclusion: Influence not significant.

1.0% = 103

1.5% = 96

Increase of the thiourea content up to:

0.25% = 100

Conclusion: Increase of the concentration gives a real improvement.

0.50% = 109

0.75% = 126

1.00% = 118

Addition of dextrine:

0% = 100

Conclusion: Addition actually causes a decline.

1% = 88

2% = 88

3% = 84

Addition of NH_4Cl :

0.0% = 100

Conclusion: The disadvantageous effect of a 1% addition is real.

0.5% = 93

1.0% = 81

Addition of NaCl :

0.00% = 100

Conclusion: Exactly like with NH_4Cl the influence of concentrations up to 0.5% is not significant.

0.25% = 99

0.50% = 93

TABLE 3.

Basic medium 2% tryptose, 0.5% neutralized ascorbic acid and 0.75% thiourea.

Addition of glycerol:

0.00% = 100

0.25% = 96

0.50% = 83

Conclusion: Addition of 0.5% is really unfavorable.

Addition of glucose:

0.00% = 100

0.25% = 97

0.50% = 88

1.00% = 102

Conclusion: The harmful effect of the addition of 0.5% disappears at a concentration of 1%.

These results show that an increase of the content of thiourea was the only quantitative change in the suspending medium that induced a significantly higher survival rate. After having verified, that a higher content of thiourea in the Str. 19 vaccine had no harmful effect whatsoever, the thiourea content of the suspending medium for the vaccine production was raised to 0.75%.

For the following 100 batches of Str. 19 vaccine the average survival rate was calculated to have risen to a good 82%.

The survival rates mentioned so far all referred to viable counts in the vacuum sealed ampoules with vaccine, ready for delivery to the veterinarian. On this same basis VERWEY, HARRINGTON and MATT (1951) obtained a survival rate of approximately 40%.

In most investigations however the survival rates are determined immediately after freeze-drying, without secondary drying. Now during the secondary drying in vacuum-desiccators over P_2O_5 and sealing of the ampoules, a further loss of viability occurs. Therefore an estimation of the survival rate in the modified suspending medium, immediately after primary drying was considered to be of interest. In this way the results obtained would be comparable with those of any other investigation.

For this reason the survival rate in 100 batches of vaccine was not only determined in the usual way in 3 finished ampoules, but also in 3 open ampoules, immediately after primary drying in the freeze dryers. The average for the latter "direct survival rates" amounted to over 88%. This means that the losses of viability caused by the actual freeze-drying process were less than 12%,

whilst the losses during secondary drying and final treatment together amounted to about 6%.

DISCUSSION.

From the results of the investigations it is apparent, that in the lyophilization of *Brucella abortus* Str. 19 high survival rates may be obtained by an adequate handling of the reducing substances added to the suspending medium and by applying them in a favorable ratio.

The observation of an unfavorable action of 1% NH_4Cl on the survival rate need not be contrary to the results of NAYLOR and SMITH (1946), as these investigators worked with another bacterium, used another protective colloid and recommended a concentration as low as 0.5%. Still in our results with NH_4Cl , we see a warning against a too lavish use of salts and buffer solutions in the suspending medium.

Of course the survival rate remains dependent on the apparatus used and on various details in the procedure, which often may be hard to describe or the influence of which may hardly be realized. Nevertheless it is probable, that on any apparatus of known fabric, with expert handling especially regarding the temperatures during freezing and drying, a high survival rate for Str. 19 will be reached when the new suspending medium described is applied properly.

With the average survival rate of 82% in the final vaccine now reached the dead ballast has been brought down substantially conform to the demands of VAN DER SCHEER and EISMA (1954).

Meanwhile HUDSON, HULSE and COLLINS (1954) also obtained survival rates considerably over 50% with Str. 19 in a suspending medium containing reducing substances. In a series of 52 batches, dried in the medium of NAYLOR and SMITH, as modified by HUTTON, HILMOE and ROBERTS, they obtained direct survival rates varying from 50–100%. No average is presented. In our 100 batches of lyophilized Str. 19 vaccine with an average direct survival rate of 88% variations occurred from 72–108%.

These large deviations from the mean might give the impression that the survival rate can fluctuate rather strongly from one batch to the other. A rough statistical calculation did show, however, that the deviations agree very reasonably with those which might

be expected on the base of a standard deviation for the viable counts of 3% of the mean, when the survival rate is actually constant. Thus it may be reasonably assumed that the actual survival rate was fairly constant and there is no motive to look for unknown factors or any technical disturbance which might have caused the deviations in the survival rate.

The wide range of variation which notwithstanding an accurate counting method appears to occur, demonstrates clearly, that it is unwarranted to draw conclusions from a single or a few viable counts and all the less so from one or a few survival rates, when the degree of accuracy of the counting method used is not known.

Summary.

For the production of lyophilized *Brucella abortus* Str. 19 vaccine a suspending medium containing 2% tryptose, 0.5% neutralised ascorbic acid and 0.75% thiourea was developed. In 100 separate batches of vaccine dried in this medium the survival rate was determined after primary drying and after secondary drying and vacuum-sealing of the ampoules.

The average survival rate immediately after primary drying was over 88%; in the finished glass sealed ampoules the average was over 82%. It is possible to obtain these results only when a freshly prepared concentrated solution of the reducing substances is added shortly before freezing to the Str. 19 suspension.

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THE ENUMERATION OF *STAPHYLOCOCCUS AUREUS* IN FOODS

by

D. A. A. MOSSEL and C. M. A. VENDRIG

(Received January 10, 1956).

The formation of an enterotoxin in perishable foods due to the prolific growth of specific types of *Staphylococcus aureus* was and remains an important cause of food poisoning (4, 1). It was therefore felt that in determining the bacteriological status of such foods, apart from counting *Salmonelleae*, coliforms and other fecal indicators, an enumeration of *S. aureus* is indispensable (2).

Various methods have been recommended for the qualitative detection and confirmation of *S. aureus* in foods. This note describes the adaptation of one of these techniques for the quantitative enumeration of those bacteria.

Agars, containing "7.5% of NaCl" (i.e. 75 g NaCl/liter medium) are the classical media for the enrichment and selective cultivation of staphylococci in general (5, 3, 2). Therefore we tried first of all to use fresh blood agar, to which 7.5% of NaCl had been added. This medium favoured both the formation of magnificently β -hemolytic colonies and appeared not to be even slightly inhibitive to the strains of *S. aureus*, tested. However, as could be expected, this medium did not suppress the growth of Bacilli, as may be seen from Table 1. Therefore, in using simple NaCl/blood/agar all colonies have to be inspected by microscope to confirm their morphology, which of course is a great disadvantage for use in routine bacteriological investigations.

Hence we decided to study the 7.5% NaCl-medium of the type, used by CHAPMAN (3) for the confirmation of primary isolates from pathological material or foods. The formula, recommended by CHAPMAN is so as to favour to the maximum extent the pigmentation of the colonies of *S. aureus*, which is very useful for our

TABLE 1.

The enumeration of staphylococci, and of bacilli, in blood agar pour plates.

Strain	Pour plate count in tryptone/dextrose/ yeast extract/agar	Pour plate count in fresh blood (10 vol. %)/agar, containing 7.5% NaCl	
		Total colonies	Hemolytic colonies
<i>S. aureus</i> 1850	1.6×10^8	1.6×10^8	0.5×10^8
<i>S. aureus</i> 1878	3.6×10^8	3.0×10^8	2.0×10^8
<i>S. aureus</i> 1910	8.1×10^8	7.3×10^8	1.3×10^8
<i>S. aureus</i> 1973	6.5×10^9	4.4×10^9	7.0×10^8
<i>B. cereus</i>	3.8×10^6	2.4×10^6	2.2×10^6
<i>B. macerans</i>	4.9×10^6	3.1×10^6	2.8×10^6

purpose as well, because it permits to differentiate immediately between true *S. aureus* and interfering Bacilli, which form colonies that are neither pigmented nor otherwise showing the characteristics of colonies of *S. aureus*.

It appeared soon that a pour plate technique concealed many of the peculiar properties of *Bacillus*- and *Staphylococcus*-colonies respectively. Therefore the drop plate method of MILES and MISRA (6) was used for the purpose in mind.

METHOD.

The medium used, had exactly the composition, recommended by CHAPMAN (3). It contained — apart from 75 g NaCl — 2 g lactose, 10 g d-mannitol, 10 g tryptone (Difco), 2.5 g yeast extract powder (Difco), 30 g gelatin and 15 g agar per liter distilled water. The pH of the medium is brought to $\text{pH} = 7.0 \pm 0.1$, with dipotassium phosphate solution. It is sterilized for 15 min. at 120°C .

Plates were poured, containing about 15 ml of medium. After solidification the plates were dried for about $\frac{1}{2}$ hour at 55°C .

Inoculation of the plates was carried out with selected standard bacteriological pipettes, producing drops of about 0.05 ml. Such dilutions were used as to produce 5-50 colonies/plate. After the drops had been deposited in the centre of the agar-surface they were — without delay — evenly spread out with the help of a sterile bended glass rod.

Plates so inoculated were dried at room temperature for 5 minutes and then incubated for 72 hours at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

RESULTS AND DISCUSSION

This drop plate technique was applied in duplicate series of tests to:

- a) five coagulase-positive *S. aureus* strains, obtained from Prof. Ruys, Laboratory of Hygiene, University of Amsterdam;
- b) one type strain of *Streptococcus faecalis*;
- c) pasteurized bulk milk, showing a plate count of the order $10^4/\text{ml}$ and an aerobic spore count of the order $10^2/\text{ml}$;
- d) the same milk, to which about 10^3 cells/ml of a *S. aureus* strain had been added.

The results obtained are recorded in Table 2. It may be concluded that the method shows a reasonable productivity as well as accuracy.

TABLE 2.

The enumeration of staphylococci on Chapman's agar, with special reference to interfering strains

Strain	Pour plate count in tryptone/dextrose/yeast extract/agar (TDYA)	Orange-yellow colony count on drop plates of	
		TDYA + 7.5% NaCl	Chapman's medium
<i>S. aureus</i> 1850	1.9×10^5	3.2×10^5	2.4×10^5
<i>S. aureus</i> 1878	6.3×10^5	5.7×10^5	4.9×10^5
<i>S. aureus</i> 1910	1.5×10^7	2.3×10^6	1.9×10^6
<i>S. aureus</i> 1973	4.6×10^5	2.2×10^5	3.7×10^5
<i>S. aureus</i> 2000	3.7×10^5	2.6×10^5	1.6×10^5
Milk + $0.6 \times 10^3/\text{ml}$ <i>S. aureus</i>	—	0.3×10^3	0.3×10^3
<i>Str. faecalis</i>	2.2×10^5	$< 10^2$	$< 10^2$
<i>B. cereus</i>	6.4×10^6	$< 2.0 \times 10^4$	$< 5.0 \times 10^3$
<i>B. macerans</i>	2.3×10^6	$< 4.8 \times 10^3$	$< 1.2 \times 10^3$

Though the counts on Chapman's medium tend to be slightly lower than on 7.5% NaCl/agar, we nevertheless prefer the former medium, since the colonies on that medium are larger and more pronounced orange-yellow.

It must further be noticed, that in a few inocula, e.g. the one of strain 1910 reported, incidentally lower drop plate counts on Chapman's agar than pour plate counts in tryptone/dextrose/yeast extract/agar (TDYA) were obtained. Since in those cases drop plate counts on 7.5% NaCl/TDYA dropped simultaneously, it is felt that this was due to dissociation phenomena in the i n o c u l u m, which do not impair the value of the m e d i u m.

The drop plate technique described has the additional advantage that it enables a very rapid and easy examination of the colonies obtained for coagulase-production.

S u m m a r y.

Staphylococcus aureus can be enumerated in foods by the use of CHAPMAN's special medium (3), under application of the drop plate technique, recommended by MILES and MISRA (6).

Colonies of *S.aureus* so obtained can easily be macroscopically differentiated from aerobic sporeformers which grow also on this medium. The colonies can be used immediately for testing their coagulase reaction.

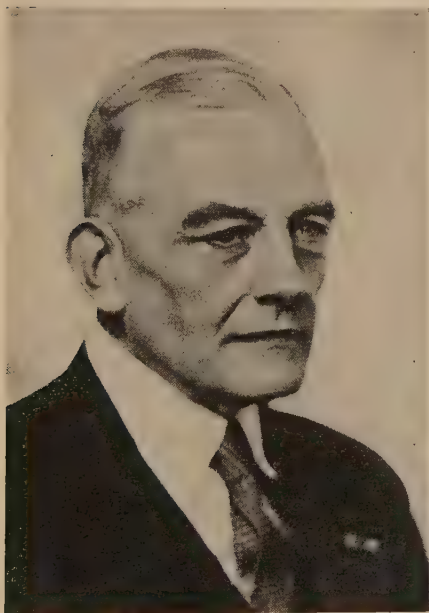
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IN MEMORIAM PROF. DR IR A. J. KLUYVER

"But it will also be evident that the promotor of 25 years ago is happy that he has had the opportunity to review these developments, and to witness the fulfillment of his prediction, made when he presented the diploma to the young Doctor, that KLUYVER would become a noble asset to science and to humanity".

This passage has been translated from a paper by G. VAN ITERSON, Jr., on "A. J. KLUYVER as a pupil, a young biologist, and a colleague", in the May 13,



Atelier Odijk, Delft

1939, issue of "Chemisch Weekblad". The issue was devoted in its entirety to Prof. Dr Ir A. J. KLUYVER, then celebrating his silver Doctor's jubilee; it contained, besides the essay of his one-time promotor, papers by two of his pupils on KLUYVER as a microbiologist, biochemist, and teacher.

Eight years later "Antonie van Leeuwenhoek" published a Jubilee Volume in honor of ALBERT J. KLUYVER in commemoration of his quarter-century professorship at the Technological University of Delft, Holland. In his dedicatory preface JAN SMIT ex-

plained that the volume had been planned

"because it is our pleasure to seize this opportunity (any opportunity!) to thank you in the name of bacteriologists, in the Netherlands and abroad, for what you have done for the advancement of our science . . .".

Manifestly, KLUYVER had done much in this respect; during those 25 years his name had become identified with some of the farthest-reaching advances in biochemistry, and his institute had acquired a world-wide reputation as one of the most important centers of microbiological research. Moreover, by demonstrating the great advantages that micro-organisms offer for studies of numerous biochemical phenomena, he had made the biochemists microbe-conscious. Hence the rapid expansion of biochemistry, resulting from the replacement of analytical-chemical studies by dynamic approaches to the chemical activities of living beings, stimulated an enormously increased interest in general microbiology. SMIT's assertion is therefore more than justified, and will be sustained by all who have the development of microbiology at heart. The profound influence of KLUYVER's work has been documented in the "A. J. Kluyver Lecture", presented before the Society of American Bacteriologists at its 1949 national meeting ("The 'Dutch School' and the Rise of General Microbiology", *Bacteriol. Revs.* **13**, 161—174, 1949).

And now, nine years after the appearance of the Jubilee Volume, "Antonie van Leeuwenhoek" publishes another issue beginning with an article on A. J. KLUYVER. But this time the occasion is not, alas, a joyous one. On the contrary, his sudden death of a heart attack during the night of May 13—14, 1956, at his home, has spread grave concern and sorrow; for among microbiologists no one was more generally admired and beloved than he. It was not only, perhaps not even primarily, as a microbiologist and biochemist that he had captured the great esteem that was accorded him; everyone who knew KLUYVER, be it ever so slightly, had become deeply impressed with his noble personality. His approach to human beings was so genuinely warm, understanding, and generous that a new acquaintance instantly experienced a strong impression of having made a personal contact of great value and depth. Thus Dr J. H. BAILEY, Secretary-Treasurer of the Soc. of Amer. Bacteriologists, who met KLUYVER during the latter's brief attendance at the national meeting in 1954, expressed his reaction to the news of KLUYVER's death in the sentence:

"I do not recall when the passing of one I knew but slightly has made me as depressed as did his";

Dr R. E. BUCHANAN, Chairman of the Judicial Commission of the

International Committee on bacteriological nomenclature, of which KLUYVER was a member, stated:

"The news of the passing of Dr KLUYVER, the Dean of Modern Bacteriologists, even yet seems almost incredible. What a loss. A great scientist and a great scientific statesman. We shall wish many times that he were with us to give wise council and leadership";

and Dr JACQUES SENEZ, who had spent a brief sojourn in KLUYVER's laboratory, felt compelled to write:

"J'avais pour le savant qu'il était une grande vénération, mais, vous qui l'avez si bien connu, vous comprendrez que je place encore plus haut dans mon souvenir l'homme admirable qu'il était. Je n'ai jamais approché personne dont la 'présence' fut plus forte et plus douce à la fois. C'est ce qui explique que tant de scientifiques qui l'ont connu, peu ou beaucoup, se réclament de lui comme d'un Maître très cher. Sa disparition crée un vide irréparable".

Such sentiments must be well-nigh universal, as those who have known him personally will be the first to agree. But even those who have heard others talk about him, or who are only familiar with his work, will realize that this is no exaggeration.

Naturally, KLUYVER exerted a profound influence on his collaborators. In the every-day life of the laboratory his beautiful qualities were always so in evidence that they will continue to be a directive force in the lives of the many Dutch and foreign students and established scientists who came to Delft to learn from the Master. Inspired by him, they created the spirit of sympathy and enthusiasm that prevailed in his institute, knowing that they could best express their devotion by endeavoring to live and work according to the example he set.

Born June 3, 1888, in Breda, Holland, ALBERT JAN KLUYVER received his higher education at the Technological University in Delft where, in 1910, he obtained the degree of Chemical Engineer, "with distinction" ¹⁾. Immediately afterwards he became assistant

¹⁾ This is the only term that the Technological University applies to indicate that a degree has been granted with recognition of unusual merit; the often encountered designations "with high" or "highest distinction" are never used in Delft.

to Prof. Dr G. VAN ITERSON, Jr., in whose laboratory he worked from 1909—1914, with an interim in 1911 that was spent in Prof. HANS MOLISCH's institute in Vienna where he had gone to broaden his training in plant biochemistry. Back in Delft he developed a method for the quantitative determination of individual sugars in mixtures, based upon the amount of CO_2 produced from aliquots by a number of specific yeasts during anaerobic incubation. Until the recent introduction of (paper) chromatographic technique this was by far the most convenient and accurate method available for the purpose. It was described in the thesis ("Biochemische Suikerbepalingen", E. J. Brill, Leiden, 1914) which, in 1914, earned him the degree of Doctor of Science, also "with distinction".

The prospects for further scientific contributions from his hand were excellent. But the outbreak of the first world war interfered temporarily; the young Doctor was induced into the Dutch army where he spent more than a year anxiously awaiting his release from military duty. After his return to civilian life he resumed his work in VAN ITERSON's laboratory till 1916, when he accepted a post as consultant for the Dutch East Indies to the Department of Agriculture, Industry, and Commerce, married Miss H. J. VAN LUTSENBURG MAAS, an able collaborator of VAN ITERSON's during her last year of study in Delft, and with his young wife moved to the colonies.

During KLUYVER's absence from Holland the authorities planned eventually to appoint him to a scientific position at the Dutch "Colonial Institute". As a preparatory move he was given the special assignment of making a detailed study of the copra fiber industry in Ceylon and Malabar. This was conducted in 1919—1920; a fully documented, 300-page report was published in 1923 ("Klappervezel- en Klappergarennijverheid", with Raden Mas ISO REKSOHADIPRODJO; de Bussy, Amsterdam). But the position at the Colonial Institute never materialized. In 1920 KLUYVER became Director of the research laboratory of a large East Indian concern.

And when, shortly thereafter, the world economic situation threatened also the stability of this industry, he was offered a professorship in Delft which, after considerable hesitation, he finally accepted.

What caused the hesitation?

The chair to which he was called was the one that the great

microbiologist, M. W. BEIJERINCK had occupied from 1895 until his retirement in 1921, and KLUYVER feared that his own limited training in microbiology might make it impossible for him to continue the work of his predecessor on a comparable level of excellence. The sponsors of KLUYVER's appointment had no such fear; they knew that he possessed the traits needed to accomplish the task successfully: a great sense of responsibility, an enormous capacity for work, an already extensive and varied factual knowledge together with a retentive memory, a fine critical ability and penetrating insight, a desire for perfection, and a pronounced interest in microbiology. Nonetheless, it would be safe to say that even his most ardent supporters could hardly have anticipated the magnitude of the advances that KLUYVER initiated after he had overcome his misgivings.

With undeviating application the young professor began by absorbing the accumulated knowledge in general microbiology, working every day from early morning till midnight and after, a schedule adhered to till death intervened. After having acquired a certain mastery of the field, and guided by the outcome of a biochemical study of a new vinegar bacterium isolated in his laboratory, he started to develop his own program. It was dictated by his desire to comprehend the almost endless diversity of biochemical transformations encountered among the numerous types of micro-organisms in terms of general principles, and led to a detailed investigation of several representative processes. Within a mere five years following his inauguration, this approach had produced results of paramount significance, for out of it had evolved the concept of the "unity in biochemistry", the essence of which was laid down in a number of publications culminating in "Die Einheit in der Biochemie" (Chem. d. Zelle u. Gewebe 13, 134—190, 1926; with H. J. L. DONKER). The fundamental thesis of this concept is that any and all biochemical processes are interpretable as the net result of more or less extended chains of simple and chemically intelligible step reactions, each one representing an (enzymatic) transfer of hydrogen from a donor to an acceptor. In due course a number of further implications of the unity concept were envisaged, yielding the equally important principles of "comparative biochemistry" which permit inferences concerning special biochemical events to be drawn from studies on processes which at first sight appeared totally unrelated. These two general ideas have

inspired many of the impressive sequences of investigations conducted in KLUYVER's laboratory during the ensuing years.

Only those who are aware of the status of biochemistry prior to 1925 can appreciate the immense clarification and order brought about by the enunciation of the above-mentioned concepts, and the impetus they gave to biochemical research; their fertilizing influence can hardly be overrated. It is not altogether superfluous to emphasize this because most younger biochemists, now engaged in elaborate studies of detail, frequently seem oblivious of the fact that the spectacular developments in modern biochemistry are firmly rooted in KLUYVER's grand generalizations and in his astute perception of their consequences.

A sound valuation of the scope and significance of this foundation can be readily acquired from a study of the classic booklet that grew out of KLUYVER's lectures before the University of London, published in 1931 by the London University Press under the title "The Chemical Activities of Micro-Organisms". Still more enlightening will be the first two chapters of "The Microbe's Contribution to Biology" (Harvard Univ. Press, 1956), in which KLUYVER has also covered the recent advances in biochemistry, and indicated how greatly these strengthen the case for unity, at the same time stressing the important role that the microbes have played in these developments.

Another major contribution of KLUYVER's, now applied on a vast scale, though often without apparent recognition of its origin ¹⁾, is the introduction of the submerged culture technique for growing molds. This development also typifies KLUYVER's characteristic approach in that it resulted from theoretical considerations. Their point of departure was the observation that the biochemical behavior of mold mycelium grown in stationary liquid cultures was extremely variable and unpredictable, even if the culture conditions had been carefully standardized. A brilliant analysis of the possible reasons for this situation led to the conclusion that the observed outcome was to be expected because the fungal mats harvested from such cultures inevitably had to consist of complex mixtures

¹⁾ See, however, the excellent account given by J. W. FOSTER in his book "Chemical Activities of Fungi", Academic Press, New York, 1949, pp. 51 ff.

of cells of heterogeneous origin possessing different biochemical properties. This follows from the fact that in a stationary culture the development in the upper parts of the mat proceeds under environmental conditions that are very different from those obtaining in the regions where the lower portions are growing, especially with respect to the concentrations of oxygen, of nutrients, and of metabolic products. And since it had long been established that the biochemical behavior of cells may be profoundly influenced by environmental conditions during growth, the erratic behavior of the mats, i.e. of the complex mixtures of cell types of different origin, was readily understandable. A more promising approach to the study of mold metabolism should therefore involve the use of physiologically uniform cells, and it was argued that these should be procurable by letting the development take place in continuously agitated liquid media.

It is an intellectual delight to read the cogent reasoning in the first publication on the subject, dating back as far as 1933 (*Biochem. Z.* 266, 68—81; with L. H. C. PERQUIN). At first the new methodology derived its interest chiefly from the possibility of investigating mold biochemistry under conditions that yielded fully reproducible results, thus permitting unambiguous interpretations. But within a decade it had become much more than a useful technique for the microbiological laboratory. On an industrial scale it was successively applied, first to the manufacture of riboflavin, then of penicillin, and still later of other antibiotics from cultures of molds and actinomycetes. Unquestionably, these industries have profited from the availability of a culture method that was the direct consequence of KLUYVER's cerebrations.

Obviously, the nearly 200 publications from his laboratory cannot be adequately reviewed here; even a mere bibliography would exceed the space limitations. The work covers a wide range of subjects in the fields of biochemistry, taxonomy, and the microbiology of foods, brines, rumen, and other specialized environments. Some of the major groups of bacteria have been subjected to monographic studies. The series of monographs on the yeasts, representing an exhaustive survey of the literature and a meticulous comparative study of the prodigious collection of yeast cultures, maintained at the Delft laboratory in collaboration with the "Central Bureau of Fungus Cultures", deserves special mention; they

now occupy a central place in the library of any one whose work may at some time involve yeasts ¹⁾).

KLUYVER's position, particularly in later years when many national and international honors were conferred on him ²⁾, contributed to his being called upon with increasing frequency to deliver special lectures. These, too, cover a great variety of subjects; they also illustrate his awareness of new developments as well as the continuing search for new relationships. One need but consider his anticipation of the use of atomic energy, expressed in his inaugural address as early as January 1922, or his forecast of the important function of CO₂, not as a metabolic waste product but as a '*spiritus vitalis*' in the metabolism of all heterotrophic organisms including man („Die Kohlensäure im Stoffwechsel der Lebewesen", Suomen Kemistilehti **12A**, 81—88, 1939). Often these lectures contain information, culled from obscure sources, that serves admirably to tie together distantly connected events. The audience listening to his address at Rutgers University on the occasion of the opening of the Waksman Institute for Microbiology must have been astonished by the wealth of detail that KLUYVER had managed to assemble in order to point out the influence that Dutch civilization had exerted on the development of the State University of New Jersey ("from Dutch Settlements to the Rutgers Institute of Microbiology"; in "Perspectives and Horizons in Microbiology"; Rutgers Univ. Press, 1955, 213—220). In the speech at the 90th

¹⁾ „Die Hefesammlung des Centraal-Bureau voor Schimmelcultures. Beiträge zu einer Monographie der Hefearten".

I. Teil. N. M. STELLING DEKKER, „Die sporogenen Hefen", Amsterdam, Drukkerij Holland, 1931.

II. Teil. 1. Hälfte. J. LODDER, „Die anaskosporogenen Hefen", Amsterdam. N.V. Noord-Holl. Uitg. Mij., 1934.

II. Teil. 2. Hälfte. H. A. DIDDENS & J. LODDER, „Die anaskosporogenen Hefen", Amsterdam, N.V. Noord-Holl. Uitg. Mij., 1942.

²⁾ KLUYVER was, for example, elected to membership in various scientific academies, including the Koninklijke Nederlandse Akademie van Wetenschappen, the Finnish Academy of Sciences, the National Academy of Sciences, U.S.A., and the Royal Society of London; and to honorary membership in the Soc. of Amer. Bacteriologists, the British Soc. for General Microbiology, and the Amer. Acad. of Arts and Sciences; he received honorary degrees from Iowa State College, the Univ. of Louvain, Rutgers University, and the Eidgenössische Technische Hochschule in Zürich; he was awarded the Emil Christian Hansen Medal in Copenhagen, and the Copley Medal of the Royal Society in London.

annual meeting of the National Academy of Sciences, U.S.A., where KLUYVER, then President of the Kon. Nederl. Akad. v. Wetensch., was the guest of honor, this feature is equally evident. Moreover, it was here that the scientist, who had been preoccupied during a lifetime with the ever-fascinating theme of diversity and unity, applied his knowledge to the problem of the freedom of science, in a manner revealing his great and humane wisdom ("An aspect of the promotion of science"; News Reports, Nat. Acad. Sci. and Nat. Res. Council 3, 33—38, 1953). Those who have known him have experienced this wisdom on many an occasion, and they will have realized that it was founded on KLUYVER's deep conviction of the supreme importance of the uniqueness of the individual, which is also the basis of ALBERT SCHWEITZER's philosophy of life.

Such lectures cannot be improvisations. But the enormous amount of work that went into their preparation can be properly assessed only by his associates, who know from experience that no effort was ever spared to present and develop an idea in the most logical, coherent, and felicitous form. If "genius is the infinite capacity for taking pains", a favorite adage of the Master often invoked to persuade his sometimes impatient collaborators that the endless revisions and polishings of a manuscript were not superfluous but effectively improved its quality, then KLUYVER must be regarded as a genius for this reason alone.

"A noble asset to science and to humanity" — verily, the prediction of his promotor has been fulfilled, for no one shall want to deny that ALBERT JAN KLUYVER has richly deserved this appellation. And, however great may be the despondency caused by his death, it will be materially assuaged by the precious memories that will always remain with those who have had the great privilege of knowing him and of experiencing his beneficent influence.

Pacific Grove, Calif.

June, 1956.

C. B. VAN NIEL

(Service d'Hygiène de la Faculté de Médecine de Bruxelles).

MODIFICATIONS REVERSIBLES DU CARACTERE DE LA REACTION DE GRAM CHEZ *E. COLI*, *PS. AERUGINOSA* ET *M. PYOGENES* VAR. *AUREUS* SOUS L'INFLUENCE DU STEARATE D'HEXAEETHYLENE GLYCOL, UN DETERSIF NON IONIQUE

par

N. DELMOTTE et A. DELMOTTE

(Reçu le 25 Octobre 1955).

Dès 1929, BÉGUET (2) étudiant les limites de la spécificité de la réaction de Gram, avait observé que deux détersifs, le pélargonate de soude et le sulforicinate de soude, ajoutés à un bouillon de culture non salé à $\text{pH} = 7$ où croissait la bactérie charbonneuse faisaient perdre à cette dernière son caractère Gram positif probablement par abaissement de la tension superficielle du milieu. Les sels biliaires, qui agissent également sur la tension superficielle, et la ribonucléase, peuvent enlever à une bactérie Gram positif son matériel ribonucléique Gram positif et cette bactérie présentera donc alors une réaction de Gram négative. Ce matériel peut être recollé grâce à certains artifices techniques simples, sur le squelette de bactéries rendues Gram négatives. Ces bactéries redeviennent donc Gram positif (1, 8-10; 13).

Il faut signaler également que Béguet (2) parvient, mais au prix de modifications morphologiques importantes, à faire acquérir au bacille dysentérique de Shiga un caractère Gram positif en incluant soit 20 % de sulfate de magnésie, soit 5 % de chlorure de sodium, soit encore 35 % de glucose au milieu de culture. Il a été montré, par ailleurs, que le bisulfite de soude, la potasse caustique et le cristal violet donnent au colibacille un caractère Gram positif (4-7).

EXPERIENCES PRELIMINAIRES.

Des détersifs ont été gracieusement mis à notre disposition par

les Usines Tensia de Liège et l'Union Chimique Belge de Bruxelles, que nous remercions ici. En voici la liste:

A. DÉTERSIFS DU GROUPE CATIONIQUE:

1. **Aminol**, pur, de formule générale:
$$\text{C}_6\text{H}_5\text{CH}_2-\text{N}(\text{R})(\text{CH}_3)_2 \cdot \text{H}_2\text{O} \text{ où R}$$

représente un groupe alkyl complexe (U.C.B.).

2. **Chlorure de cétypyridinium** (Tensia).
3. **Chlorure de cétyldiméthylbenzylammonium** (Tensia).

B. DÉTERSIFS DU GROUPE NON-IONIQUE.

- I. de formule générale $\text{R-O}(\text{C}_2\text{H}_4\text{O})_n\text{H}$.
4. **alkylpolyéthylène-glycol**: Emullat S 25 (U.C.B.).
5. **éther de polyéthylène-glycol**: Emullat CO 10 (id.).
6. **alkyl-aryl-éther-polyéthylène-glycol**: Emullat 29 (id.).
7. **alkyl-aryl-éther-polyéthylène-glycol**: Ucépál DC (id.).
- II. de formule générale: $\text{R-COO}(\text{C}_2\text{H}_4\text{O})_n\text{H}$.
8. **ester de polyéthylène-glycol et d'acide gras**: Emullat 06 (U.C.B.).
- III. de formule générale $\text{R}[(\text{C}_2\text{H}_4\text{O})_n\text{H}]_3$.
9. **éther polyéthylène-glycol d'un glycéride d'acide gras**: Emullat R 40 (U.C.B.).
- IV. condensat d'éthanolamine sur acide gras.
10. **condensat de mono-éthanolamine sur acide gras**: Tensomel P.E.11 (Tensia).
11. **condensat de diéthanolamine sur acide gras**: Tensomel P.E. 22 (Tensia).
- V. condensat d'oxyde d'éthylène.
12. **condensat d'oxyde d'éthylène sur alcool oléique**: Tensolase D 30 (Tensia).
13. **condensat d'oxyde d'éthylène sur alcool $\text{C}_{16} - \text{C}_{18}$** : Tensolase D 60 (Tensia).
14. **condensat d'oxyde d'éthylène sur alcool $\text{C}_{12} - \text{C}_{14}$** : Tensolase DL 8 (Tensia).
15. **condensat d'oxyde d'éthylène sur nonylphénol**: Tensophène D 42 (Tensia).
- VI. Stéarate d'hexaéthylène glycol.
16. à 100 % de concentration: Belsam III (U.C.B.).
- 16a. à 50 % de concentration: Belsam III/50 (id.).
- VII. éther de polyéthylène-glycol.
17. **Base LP 12** (U.C.B.).
- VIII. éther sulfosuccinate basique.
18. **Synkapon** (U.C.B.).

C. DÉTERSIFS DU GROUPE ANIONIQUE.

19. **sulfate sodique d'alcool $\text{C}_{12} - \text{C}_{14}$** : Tensopol U.S.P. (Tensia).
20. **sulfate magnésique d'alcool $\text{C}_{12} - \text{C}_{14}$** : Tensopol MG (Tensia).
21. **sulfate ammonique d'alcool $\text{C}_{12} - \text{C}_{14}$** : Tensopol N (Tensia).

22. sulfate sodique d'alcool $C_{16} - C_{18}$: Tensadine D 119 (Tensia).
23. sulfate sodique d'alcool $C_8 - C_{10}$: Tensatil DN 225 (Tensia).
24. sulfate sodique d'éther polyglycolique: Tensagex DL 12 (Tensia).
25. sulfonate sodique de dodécylbenzène: Tensaryl 80 B (Tensia).

MATERIEL.

- une souche de *Micrococcus pyogenes* var. *aureus*, issue de lésion cutanée;
- une souche d'*Escherichia coli*, issue d'ulcère de jambe;
- une souche de *Pseudomonas aeruginosa*, issue d'ulcère de jambe;
- une solution du détersif choisi à 5 ‰ en eau physiologique;
- les réactifs du Gram.

Technique.

0,2 cc d'une solution à 5 ‰ en eau physiologique du détersif choisi sont ajoutés à 0,2 cc d'un bouillon de culture de 24 heures d'*E. coli*, de *Ps. aeruginosa* ou de *M. pyogenes* var. *aureus*.

Après dix à quinze minutes de contact, la réaction de Gram est pratiquée et comparée à une réaction faite à partir d'une culture non traitée de la bactérie correspondante.

Resultats.

La technique de la réaction de Gram doit être aussi scrupuleusement suivie que possible, notamment en ce qui concerne le temps de décoloration, car la notion de "prend" ou "ne prend pas" le Gram est relative (12) et dépend du temps de décoloration. Il est bien connu en effet (14) que si la décoloration est poursuivie longtemps, pendant une heure par exemple, de nombreux germes prenant le Gram dans des conditions ordinaires seront décolorés. La vitesse de décoloration varie suivant l'espèce bactérienne.

C'est donc dans des conditions techniques précises qu'il faut distinguer (12) une coloration violet-rouge ou violet-bleu correspondant à une réaction positive, une coloration gris clair violacée à une réaction légèrement positive et la coloration rouge de contraste à une réaction négative.

Action vis-à-vis de *E. coli*.

a. Apparition d'un caractère Gram positif:

1. quatre détersifs non-ioniques: le Tensomel P.E. 22, la base LP 12, le Belsam III et le Synkapon.

2. trois détersifs anioniques: le Tensopol N, le Tensopol U.S.P. et le Tensatil D.N. 225.

b. Apparition d'un caractère Gram légèrement positif:

1. quatre détersifs non-ioniques: le Tensophène H 10, l'Ucépal DC, le Belsam III/50 et la Tensolase D 30.

2. un détersif anionique: la Tensadine D 119.

c. Intensification de la coloration de contraste chez les bactéries traités:

Deux détersifs cationiques: l'Aminol (qualité technique) et le chlorure de cétylpyridinium.

Action vis-à-vis de *Ps. aeruginosa*.

a. Apparition d'un caractère Gram positif:

1. cinq détersifs non-ioniques: le Tensomel P.E. 22, le Belsam III, le Synkapon, le Tensophène H 10 et l'Ucépal DC.

2. deux détersifs anioniques: le Tensopol N et le Tensatil DN 225.

b. Intensification de la coloration de contraste chez les bactéries traités:

Un détersif cationique: l'Aminol (qualité technique).

Action vis-à-vis de *M. pyogenes* var. *aureus*.

a. Apparition d'un caractère Gram négatif.

Seul, le Belsam III/50 est actif.

b. Apparition d'un caractère Gram positif plus marqué chez les bactéries traitées que chez les bactéries témoins.

1. quatre détersifs non-ioniques: la base L.P. 12, le Belsam III, l'Ucépal DC et la Tensolase D 30;

2. deux détersifs anioniques: le Tensopol U.S.P. et la tensadine D 119.

3. deux détersifs cationiques: l'Aminol (pur et qualité technique) et le chlorure de cétylpyridinium.

Conclusion.

Les modifications du caractère de la réaction de Gram paraissent, dans les conditions de l'expérience, plus faciles à obtenir avec *E. coli* qu'avec *Ps. aeruginosa*. A quelques exceptions près, les mêmes détersifs appartenant aux groupes non-ioniques et anioniques, sont actifs.

M. pyogenes var. *aureus*, dans les conditions expérimentales, ne

devient Gram négatif que sous l'action du stéarate d'hexaéthylène glycol à 50 % ou Belsam III/50. Par contre, le caractère Gram positif du staphylocoque est exagéré par plusieurs détersifs appartenant aux groupes anionique, non-ionique et cationique.

Il semble que l'on ne puisse relier l'action particulière d'un détersif à sa formule chimique ou à son appartenance à un des groupes anionique, cationique ou non-ionique. Il est cependant permis de constater que, dans l'ensemble, les détersifs cationiques exagèrent la positivité initiale de la réaction de Gram ou la coloration de contraste des bactéries à Gram négatif.

MODIFICATIONS REVERSIBLES DE LA REACTION DE GRAM SOUS L'INFLUENCE DU STEARATE D'HEXAÉTHYLÈNE GLYCOL.

Les résultats obtenus à l'aide du stéarate d'hexaéthylène glycol à 100 % (Belsam III) et à 50 % (Belsam III/50) paraissent, à première vue, peu conciliables.

En effet, le stéarate d'hexaéthylène glycol à 100 % conférait au *Ps. aeruginosa* et au *E. coli* un caractère Gram positif et rendait le caractère Gram positif du staphylocoque beaucoup plus marqué que celui du témoin non traité. Au contraire, le stéarate d'hexaéthylène glycol à 50 % ne modifiait pas le caractère Gram négatif du *Ps. aeruginosa*, déterminait l'apparition d'une coloration Gram légèrement positive pour *E. coli* et négativait le caractère Gram positif du *M. pyogenes* var. *aureus*!

Dans le but d'éclaircir ces contradictions apparentes, de nouvelles recherches furent entreprises en modifiant le temps de réaction sans toucher aux autres conditions expérimentales.

C'est ainsi qu'il a pu être établi que sous l'action du stéarate d'hexaéthylène glycol tant à 100% qu'à 50%, le staphylocoque dont le caractère Gram positif demeure tout d'abord inchangé, passe par un stade Gram négatif pour redevenir ensuite Gram positif avec, souvent un caractère de positivité plus marqué que chez le témoin.

On peut saisir les mêmes modifications, mais en sens inverse avec *E. coli* et *Ps. aeruginosa*: caractère Gram négatif inchangé, passage par une réaction Gram positive et Gram légèrement positive et retour à un caractère Gram négatif avec coloration de contraste souvent plus marquée que chez le témoin.

Toutefois, si ces modifications ont pu être répétées au cours d'expériences de contrôle, elles ont toujours été de durée assez courte et sont apparues après des intervalles de temps variables.

Le caractère individuel des cellules bactériennes joue donc un rôle certain dans le moment d'apparition des modifications du Gram.

Des essais de remise en contact ou de repiquage après centrifugation lorsque la modification du caractère du Gram était obtenue furent pratiqués en vain: au cours du temps nécessaire à la centrifugation, la coloration de Gram avait repris son caractère original.

En conséquence, le stéarate d'hexaéthylène glycol (Belsam III et Belsam III/50) est capable de modifier et d'une façon réversible, le caractère de la réaction de Gram tant pour les bactéries à Gram négatif: *E. coli* et *Ps. aeruginosa* que pour les cocci à Gram positif: *M. pyogenes* var. *aureus*.

CONCLUSION.

Les modifications du caractère du Gram obtenues à l'aide de détersifs variés et le fait que dans le cas du stéarate d'hexaéthylène glycol ces réactions sont réversibles nous incitent à croire que dans certaines circonstances, la réaction de Gram dépend d'un état physico-chimique déterminé par la mise en équilibre avec le milieu extérieur (2, 3, 11).

Résumé.

Certains détersifs appartenant aux groupes anioniques et non-ioniques font acquérir au *E. coli* et au *Ps. aeruginosa* un caractère Gram positif. Seul, le stéarate d'hexaéthylène glycol, détersif non-ionique négative la réaction de Gram du *M. pyogenes* var. *aureus*. Les modifications du caractère de la réaction de Gram du *E. coli*, du *Ps. aeruginosa* et du *M. pyogenes* var. *aureus* obtenues à l'aide du stéarate d'hexaéthylène glycol sont réversibles en ce sens qu'après un certain temps de contact la bactérie reprend sa réaction primitive. Ainsi donc, pour le staphylocoque, le caractère Gram positif demeure tout d'abord inchangé, puis devient Gram négatif pour reprendre ensuite un caractère Gram positif. Les variations sont en sens inverse pour *E. coli* et *Ps. aeruginosa*.

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DIFFERENTIATION OF SPINDLE, CHROMOSOMES AND CENTRIOLE IN THE SPORE OF *BACILLUS MEGATHERIUM*

by

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When spores are formed in *Bacillus megatherium* either small cells are produced by division of the vegetative cells and the entire protoplast of each small cell is transformed into a spore (fig. 1a) or a single large vegetative cell is transformed into a spore (fig. 1b). In the latter instance, a conspicuous spindle body and other cytoplasmic material remain outside the spore wall. The different structures identified as spindle (sp), cytoplasm (c), nucleus (n) and chromosomes (ch) by YUASA (5) are visible in the spore and have been described by other workers, ROBINOW (4), DELAMATER (1), and FITZ-JAMES, ROBINOW and BERGOLD (2), but not with the present designations. A variety of fixatives and stains were employed with the results shown in table 1. Comparison of 3 (the numbers refer to the different experiments described in table 1) and 6 make it clear that the spore does not stain with gentian violet after treatment with Carnoy fluid unless the fixation with Carnoy is followed by a treatment with 10 per cent perchloric acid. Experiment 8 shows that the chromosomes are Feulgen-positive while the spindle is Feulgen-negative. Occasionally, the spindle may stain a reddish violet after short treatment with hydrochloric acid, but then the cytoplasm is also faintly stained. Comparison of experiments 1, 2 and 3 with 4, 5, 6, 8, 9 and 10 show that some treatment following fixation is required to make the spore stainable. When dried preparations were treated with KOH or KSCN a small dense structure is revealed following destruction of the spindle. It is inferred that a centrosome containing a centriole is embedded in the spindle

TABLE 1.

Summary of Experiments.

Spindle = sp; Nucleus = n; Cytoplasm = c.

Fixation	Pretreatment	Staining	Results
1) Drying (without heat)	—	1% gentian violet solution	spores unstained
2) Drying (without heat)	—	giemsa solution	spores unstained
3) Drying (without heat) followed by fixation with Carnoy fluid (3 : 1) for several minutes	—	1% gentian violet solution	spores unstained
4) Drying (without heat)	10% perchloric acid for 20 minutes at room temperature	1% gentian violet solution	sp stains deeply and n moderately.
5) Drying (without heat)	10% perchloric acid for 20 minutes at room temperature	mounted in water	sp and n are clearly visible
6) Drying (without heat) and then Carnoy fluid (3 : 1) for several minutes	10% perchloric acid for various times (10 m., 20 m., 30 m., 1 h., 3 h.)	1% gentian violet solution	sp stains deeply (Fig. 3).
7) Drying (without heat)	10% perchloric acid for various times (10 m., 20 m., 30 m., 1 h., 3 h.)	1% gentian violet solution	sp and n stain deeply, but c and n often destroyed when treated with perchloric acid for more than 30 minutes (fig. 4).
8) Drying (without heat)	1 N HCl for 5-6 minutes at 60°C.	Feulgen's nucleal staining	sp is negative, but n is positive.

TABLE 1 (continued).

Fixation	Pretreatment	Staining	Results
9) Drying (without heat) and then Carnoy fluid (3 : 1) for several minutes	1 N HCl for 10 m., 20 m., 30 m., or 1 h. at room temperature	1% gentian violet solution	sp and n stain. When treated with HCl for 1 h. a centriole appears instead of sp (Fig. 5).
10) Drying (without heat)	4% HNO ₃ for 10 m., 20 m., or 30 m.	1% gentian violet solution	sp and n stain, but they are often destroyed when treated with HNO ₃ for more than 1 h.
11) Drying (without heat)	1% KOH for 10 m., 20 m., 30 m., 1 h. or 3 h.	1% gentian violet solution	spores unstained. The n of the vegetative cell is clumped. sp remains and often shows the centriole (Fig. 6).
12) Drying (without heat)	1% KSCN for 10 m., 20 m., 30 m., 1 h., or 3 h.	1% gentian violet solution	spores unstained. The vegetative cell often shows the centriole; n dissolves.
13) Drying (without heat)	5% H ₂ SO ₄ for 10 m., 20 m., 30 m., 1 h. or 3 h.	1% gentian violet solution	spores unstained. sp stains occasionally (Fig. 8).
14) Drying (without heat)	10% perchloric acid for 20 m. and 1% KOH for 10 m., 20 m., or 30 m.	1% gentian violet solution	sp and n stain. Sometimes they are destroyed (Fig. 9).
15) Drying (without heat)	1% picric acid for 10 m., 20 m., 30 m., 1 h. or 3 h.	1% gentian violet solution	spores unstained.
16) Drying (without heat)	1% chromic acid for 10 m., 20 m., 30 m., 1 h. or 3 h.	1% gentian violet solution	spores unstained.
17) Drying (without heat)	1% potassium chromate for 10 m., 20 m., 30 m., 1 h. or 3 h.	1% gentian violet solution	spores unstained.

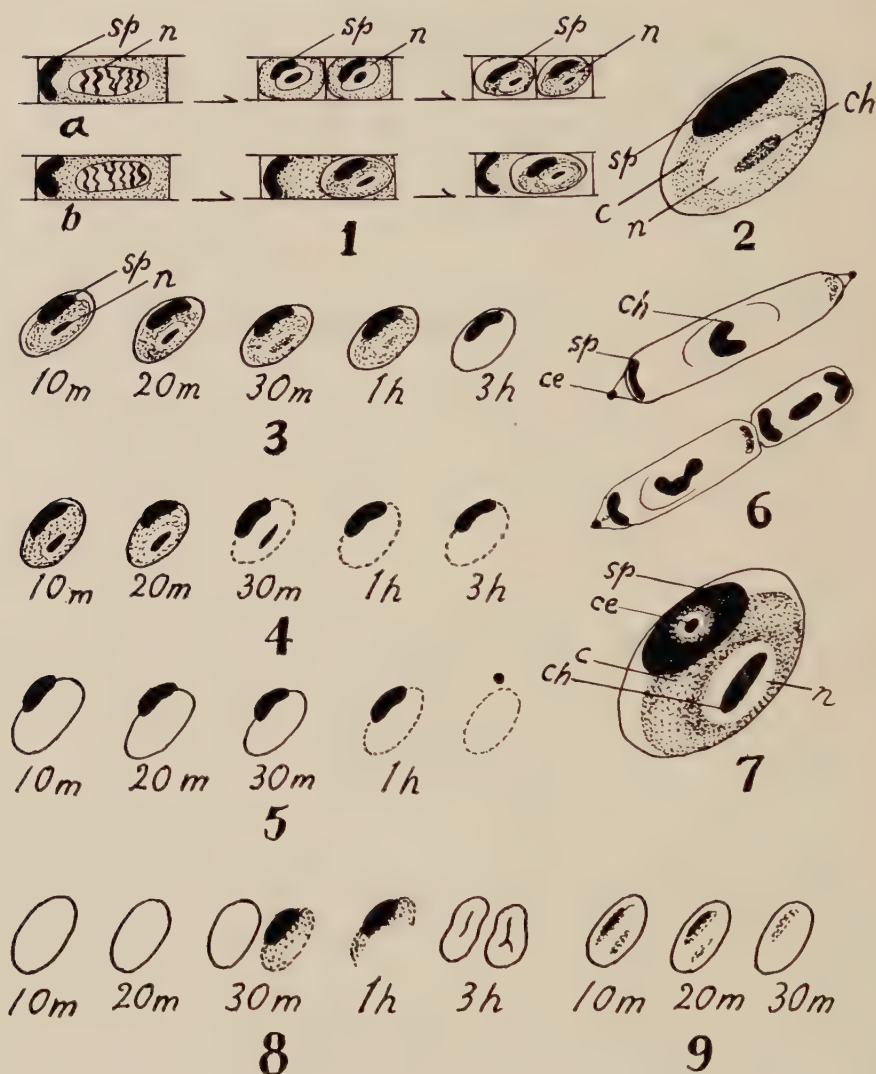


Fig. 1. (a) The entire contents of the small vegetative cells are transformed into spores. (b) When the spore is formed from a large vegetative cell some portion of the spindle and the cytoplasm is left outside the spore. n, nucleus; sp, spindle.

Fig. 2. Schematic representation of the inferred structure of the spore. c, cytoplasm; ch, chromosomes.

as shown in figure 7. The structure of the spindle in *Bacillus megatherium* closely resembles that inferred for the yeast cell (3).

S u m m a r y.

Methods of fixation and staining which make it possible to differentiate the spindle, the chromosomes and the nucleus of *Bacillus megatherium* have been defined. The spindle is Feulgen-negative while the chromosomes are Feulgen-positive. The spindle is rigid and contains a centrosome and its centriole.

The work was done at Southern Illinois University in the laboratory of Dr CARL C. LINDEGREN whom the author wishes to thank for his valuable suggestions and criticisms. He is also indebted to Dr MAURICE OGUR for helpful suggestions.

Fig. 3. Schematic drawings of spores stained with 1 per cent gentian violet after fixation with Carnoy fluid for several minutes and treatment with 10 per cent perchloric acid at room temperature for the various times indicated in the drawing.

Fig. 4. Dried preparations of spores treated for various times with 10 per cent perchloric acid and then stained with 1 per cent gentian violet solution. Figures 3 and 4 show the comparative effects of pretreatment with Carnoy fixation. Cells shown in figure 3 were fixed with Carnoy while those shown in figure 4 were not.

Fig. 5. Dried preparations of spores fixed with Carnoy fluid for several minutes and treated for the indicated times with 1 N HCl and stained with 1 per cent gentian violet. The dense structure remaining after treatments longer than 1 hour with 1 N HCl is referred to be the centriole.

Fig. 6. Vegetative cells stained with 1 per cent gentian violet solution after treatment with 1 per cent KOH. The centriole (ce) is associated with the spindle.

Fig. 7. A schematic drawing of the inferred structure of the spore.

Fig. 8. Spores stained with 1 per cent gentian violet after treatment with 5 per cent H_2SO_4 for the times indicated.

Fig. 9. Dried preparations of spores treated with 10 per cent perchloric acid and with 1 per cent KOH for the times indicated and then stained with 1 per cent gentian violet.

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THE ACTIVITY OF SOME ANTIBIOTIC COMBINATIONS ON *SALMONELLA*

by

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(Received April 20, 1956).

At the moment therapy of salmonellosis in men is most adequately carried out by a treatment with the antibiotic chloramphenicol. The cure obtained with this drug is sometimes incomplete owing to the survival of bacteria which either cause a re-infection of the patient after treatment has been stopped or may render him a carrier.

In the search of more potent weapons against infections due to the various *Salmonella* species the aim should therefore be to find agents which cause a complete eradication of bacteria under in vitro as well as under in vivo conditions. In the following a number of in vitro experiments are reported to find out if such an effect might be expected to arise from combinations of antibiotics.

The degree of bactericidal action of a drug in vitro can be determined by applying either a technique of subculturing from bacterial suspensions in graded amounts of antibacterial substances, or by ELEK and HILSON's variant of LEDERBERG's replica technique (1, 4). The latter method was chosen by us, because in earlier experiments we found that this technique allows a rapid orientation on the effect of a relatively wide range of concentrations of antibiotics acting alone or in combination upon a large number of strains. This method has been more extensively described in an earlier paper (5).

The antibacterial effects of the antibiotics streptomycin, chloramphenicol, tetracycline, polymyxin B and neomycin were investigated. These antibiotics were applied to homogeneously implanted

¹⁾ With the technical assistance of Miss M. J. WISSE.

plates in paper discs containing 5 μ g of polymyxin and 10 μ g of the other four antibiotics. Replica's were taken of the primary plates after incubating them for 18 hours at 37°C. The effect of a single antibiotic was read as bactericidal if the replica of the zone of inhibition showed after incubation 0 to 6 colonies, as partly bactericidal if these colonies numbered 5 to 25, and as bacteriostatic if more than 25 to numerous colonies had developed.

RESULTS.

Up to 72 strains of *Salmonella* were tested, comprising 17 of *S. typhosa*, 20 of *S. paratyphi* B, 19 of *S. typhimurium*, and 16 of *S. bareilly*, *S. manhattan*, *S. dublin*, *S. heidelberg* and some other species. The different species exhibited in general the same pattern in their response to the action of antibiotics singly and in combinations, which allows the results of the experiments to be regarded as typical for the whole genus *Salmonella*.

The results of the experiments are summarized in table 1.

TABLE 1.

Antibacterial action of some antibiotics on *Salmonella* strains.

Antibiotic	Number of strains investigated	Type of antibacterial action in strains		
		bactericidal	partly bactericidal	bacteriostatic
Streptomycin	28	3	12	13
Chloramphenicol	72	0	2	70
Tetracycline	24	0	0	24
Polymyxin B	72	39	?	?
Neomycin	72	13	54	5

It appears from the table that the action of chloramphenicol and of tetracycline under the experimental conditions was preponderantly bacteriostatic. Less clear cut results were obtained with streptomycin and with neomycin which substances varied in their action from definitely bactericidal for some strains to no more than bacteriostatic for other ones. The reading of the results obtained with polymyxin was often impaired by the small zone of inhibition which this antibiotic produced (cf. Fig. 1). In all cases where interpretation was possible the action of this antibiotic appeared to be bactericidal.

Interaction between the antibiotics was assessed by a double test (1). Antagonism was present if the bactericidal or partly bactericidal action of an antibiotic was found to be lost in the presence of a second. The test for synergism or for neutrality was carried out by applying to a homogeneously inoculated plate two paper discs, each with one antibiotic, in such a way that the zones of

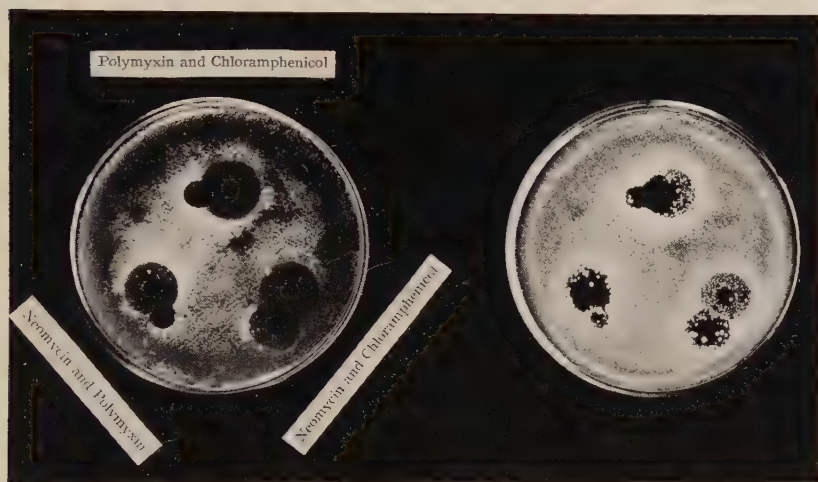


Fig. 1. Interaction between chloramphenicol, polymyxin and neomycin with *Salmonella typhosa* as test organism. Synergism is shown between polymyxin B and chloramphenicol since subinhibitory concentrations of polymyxin become bactericidal where this antibiotic mixes with the bacteriostatic substance chloramphenicol. Interaction between the combinations neomycin-polymyxin B and neomycin-chloramphenicol is absent in this picture.

inhibition overlapped. Synergism was read if the bactericidal action of one drug extended where it met the second one, indicating that a range of subinhibitory concentrations of the former drug became bactericidally active in the presence of a range of (inhibitory) concentrations of the second one (compare Fig. 1).

The results of these experiments are represented in table 2.

It is evident from the table that, with a few exceptions, no interaction is shown between streptomycin and chloramphenicol and between streptomycin and tetracycline.

The results with respect to the combinations of neomycin with chloramphenicol and with polymyxin are less clear cut; antagonism seems to be the prevailing type of interaction between neomycin and chloramphenicol, synergism that between neomycin and poly-

TABLE 2.

Interaction between pairs of antibiotics with *Salmonella* as test organism.

Pair of antibiotics	Number of strains tested	Type of interaction		
		synergism	neutrality	antagonism
Streptomycin + chloramphenicol	24	1	20	3
Streptomycin + tetracycline	24	1	23	0
Chloramphenicol + polymyxin B	72	70	2	0
Chloramphenicol + neomycin	72	7	23	42
Polymyxin + neomycin	72	38	33	1

myxin. Between the latter pair of antibiotics synergism has also been reported to occur with *Shigella*-organisms (7).

The main result obtained in the experiments is the synergistic interaction shown by chloramphenicol and polymyxin B which, using our method of experimentation, appeared in no less than 70 out of 72 strains of *Salmonella* investigated. Under the conditions of our experiments the synergism noticed was rather considerable, since the extension of bactericidal action of polymyxin B in the zone of chloramphenicol inhibition is relatively wide (see top of Fig. 1). Compared with this, synergism between neomycin and polymyxin was less frequently encountered and, if occurring, only relatively weak.

DISCUSSION.

As already remarked in the beginning, an ideal drug against salmonellosis should be bactericidally active and not, such as with chloramphenicol, act only by bacteriostasis.

The combination of chloramphenicol with polymyxin B appeared in our hands to be highly bactericidal in vitro, and this antibiotic pair might therefore be tried for a more radical therapy of salmonellosis in patients.

In the literature we found one report of a patient who, after developing a chronic carrier state which appeared to be refractory to therapy with chloramphenicol alone, responded favourably to combined antibiotic treatment with this antibiotic plus polymyxin

(2). Although it is tempting to speculate about this encouraging result, some caution should be made in view of the results of some other experiments reported in literature. JAWETZ *et al.* (2, 3), for instance, encountered synergism between the pair of antibiotics in question less frequently with *Salmonella* than e.g. with coliform organism. In addition PRICE *et al.* (6), experimenting also with *Salmonella*, found the additive or synergistic effect of combining polymyxin with several other antibiotics to be only poorly correlated with the results of treatment of experimental infections. Therefore, our results do not allow predictions on a possibly synergistic effect of the polymyxin B-chloramphenicol combination in man and animals. Experiments *in vivo* are required to settle this question definitely.

S u m m a r y.

The effects of some antibiotics acting alone or in combination upon 72 strains of various *Salmonella* species were investigated using ELEK and HILSON's variant of LEDERBERG's replica technique.

Chloramphenicol and tetracycline were found to act mainly bacteriostatically; streptomycin and neomycin varied in their action from bacteriostatic for some strains to bactericidal for other ones. The action of polymyxin B appeared to be more especially bactericidal.

But for a few exceptions no interaction was found between the combinations streptomycin-chloramphenicol and streptomycin-tetracycline. The pair neomycin-chloramphenicol was often antagonistic; neomycin-polymyxin B, on the other hand, prevalingly synergistic. Under the conditions of experimentation a pronounced synergism was noticed between polymyxin B and chloramphenicol.

The prospects of the latter synergistic combination as a possibly more radical means of combating *Salmonella* infections are shortly discussed.

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THE GROWTH OF *SACCHAROMYCOPSIS* *GUTTULATA*

by

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During a study of the yeasts occurring in the intestinal tracts of certain mammals, attention was drawn to the repeated appearance in the intestines of rabbits of a yeast identified as *Saccharomyopsis guttulata* (Robin) Schiönning. Since this yeast had not been grown satisfactorily in artificial culture its physiological characters were of some interest.

In 1903 SCHIÖNNING established the genus *Saccharomyopsis* with the following diagnosis: Budding yeast cells with endospores. The spore has two cell membranes and germinates by budding. SCHIÖNNING placed two species in this genus, *Saccharomyopsis capsularis* the type species and *Saccharomyopsis guttulata*. Subsequently GUILLIERMOND (1908) transferred *Saccharomyopsis capsularis* to the genus *Endomyces*. *Saccharomyopsis guttulata* has been retained as a separate species by STELLING-DEKKER (1931) and LODDER and KREGER-VAN RIJ (1952) owing to lack of knowledge of its physiological characteristics.

Since its description by ROBIN (1853) a number of attempts have been made to isolate and maintain this organism but with little success. WILHELM (1898) obtained growth of *Saccharomyopsis guttulata* in a medium consisting of an acidified extract of hay with glucose added. COCHET (1940) observed sporulation in rabbits faeces which had been moistened and allowed to dry slowly. LODDER and KREGER-VAN RIJ (1952) obtained good growth in malt extract at pH 2.5 when inoculated with stomach contents of rabbits, they

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did not however succeed in transferring from the primary culture into a second medium of the same composition.

EXPERIMENTAL.

Since WILHELM (1898) had reported growth in a medium containing hay extract, a medium was prepared using an infusion of grass enriched with glucose and Difco autolysed yeast. No growth was obtained in this medium. A similar type of medium containing a commercial vegetable extract in place of the grass infusion was also tried without success.

As *Saccharomycopsis guttulata* has an extremely restricted habitat, occurring commonly only in the stomach of rabbits, it was thought possible that some growth factor required by this organism was present in the intestine. Consequently a medium was prepared as follows:

Stomach contents from two rabbits were suspended in 500 ml of distilled water and autoclaved for 10 minutes at 15 lb. The resulting solution was filtered through gauze and paper and the filtrate made up to 500 ml. To this solution was added 20 g glucose and 1.25 g Difco yeast extract. The medium was adjusted to pH 3.5 and sterilized by autoclaving for 15 minutes at 15 lb.

Using this medium several strains of *Saccharomycopsis guttulata* were successfully carried through a large number of subcultures.

Dialysis of the stomach extract for 24 hours against distilled water did not alter its growth promoting activity. However removal of proteins by precipitation with ammonium sulphate, followed by dialysis to remove the salt, completely destroyed the activity of the solution for *Saccharomycopsis guttulata*.

To obtain a bacterial free culture of the organism, 0.1 mg/ml of aureomycin was added to the medium. If contaminating yeasts were present they could not be separated and overgrew *S. guttulata*. Tubes were inoculated with 2 or 3 loopsful of the stomach content and incubated at 37°C. A lag period of from 12–18 hours was observed but growth was very heavy with considerable gas production after 48 hours. The organism remained viable for up to ten days in this medium but better growth was obtained by inoculating fresh media with 2–3 drops of a well grown culture at weekly intervals.

Attempts were made to grow the yeast on the stomach extract medium solidified with 2% agar but with little success. An extremely heavy surface inoculation with a well grown broth culture

of the organism gave rise to small colonies approximately 0.5–2.0 mm, in diameter after 3–4 days but these did not increase in size even on prolonged incubation (Fig. 1). Pour plates made from a broth culture gave no growth.

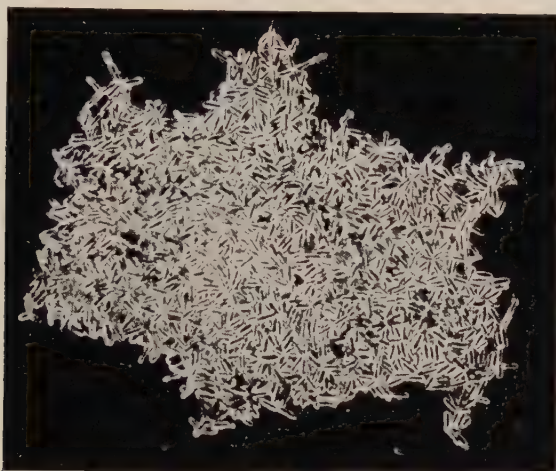


Fig. 1. Colony of *Saccharomycopsis guttulata*. Phase contrast. Magnification approximately $\times 80$.

Fermentation reactions were examined by replacing the glucose in the stomach extract broth with the appropriate sugar and sterilizing by filtration. No acid indicator was added to the medium, gas production alone being used as the criterion of fermentation. Control tubes containing broth with no added sugar were inoculated in parallel with the test sugars.

Carbon assimilation reactions were tested in media similar to those used for the fermentations. No growth occurred in any of the control tubes.

Nitrate utilisation as tested by the method laid down by LODDER and KREGER-VAN RIJ (1952) was negative. It could not be determined by methods similar to those used to study carbon assimilations owing to the high proportion of protein material in the complex media required for the growth of *S. guttulata*.

Sporulation was observed on both Gorodkowa agar and turnip plugs at 18°C., 2–3 weeks after a heavy inoculation with a freshly isolated culture. The power to form spores appeared to be lost after successive transfers through artificial media.

Description of *Saccharomycopsis guttulata*

Cells from young cultures oval to cylindrical $4-7 \times 11-21 \mu$ growing singly and in pairs or large groups (Fig. 2). No vacuoles in young cultures but 2-3 large vacuoles are present in older cells.

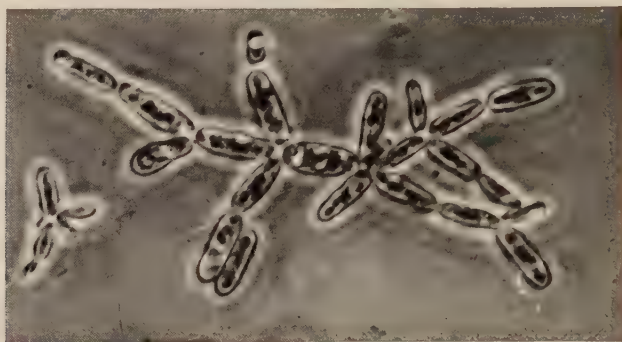


Fig. 2. *Saccharomycopsis guttulata*. Unstained preparation from a broth culture. Phase Contrast. Magnification approximately $\times 400$.

Broth culture: Growth only at 37°C . and pH 2.0-4.5 Heavy white sediment but no ring or pellicle present. Considerable gas is formed in media containing glucose.

Growth on malt agar: Poor, colonies after 3 days are minute 0.5-2.0 mm in diameter, irregular, grey-white, with a matt surface. No increase in size after prolonged incubation. No growth in deep culture.

Sporulation: Spores large and oval 1-3 per ascus (Fig. 3). No apparent conjugation prior to ascus formation.



Fig. 3. Ascospores of *Saccharomycopsis guttulata*. Unstained preparation from Gorodkova agar. Phase Contrast. Magnification approximately $\times 1700$.

Fermentation:

Glucose	+	Sucrose	+
Maltose	—	Galactose	—
Lactose	—	Raffinose	—

Carbon assimilation:

Glucose	+	Sucrose	+
Maltose	—	Galactose	—
Lactose	—	Raffinose	—

Nitrate utilisation: This could not be determined.

DISCUSSION.

Since the methods used to determine the fermentation and assimilation patterns of this organism were different from those of LODDER and KREGER-VAN RIJ (1952), the results reported above are provisional only and must be confirmed by the standard procedures when the growth requirements of *Saccharomycopsis guttulata* are more fully understood. Although the ability to utilise nitrate could not be studied satisfactorily it seems improbable that an organism with such complex requirements could use this substance as a sole source of nitrogen. This assumption is supported to some extent by the fact that the growth promoting effect of the stomach extract was removed by precipitating the proteins contained in it.

The poor growth on solid media may be due to some difficulty in obtaining nutrients or to a toxic factor in the agar used to solidify the medium. More work is necessary on this aspect of the growth of this organism.

It is noteworthy that the diagnostic requirements for the genus *Saccharomyces* are, so far as is known, fulfilled by *Saccharomycopsis guttulata*. The possibility of discarding the genus *Saccharomycopsis* and placing *Saccharomycopsis guttulata* in to the genus *Saccharomyces* has been considered but is thought inadvisable until the growth requirements of the organism are more fully elucidated.

Summary.

A medium containing an extract of rabbit stomach contents for the growth of *Saccharomycopsis guttulata* (Robin) Schiönning is described. Growth characteristics and tentative fermentation and carbon assimilation patterns are reported. Nitrate assimilation

could not be determined with the media available. Sporulation has been observed on artificial media.

The growth promoting substance is heat stable and non dialysable, it appears to be destroyed by precipitation of protein from the active extract solution.

The possibility of transferring *S. guttulata* to the genus *Saccharomyces* and discarding the genus *Saccharomyopsis* is discussed.

A culture of *Saccharomyopsis guttulata* has been deposited at the Centraalbureau voor Schimmelcultures, Delft.

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(From the Laboratory of Parasitology of the University, Parasitological
Department of the Institute of Tropical Medicine at Leyden).

THE PERSISTANCE OF *TOXOPLASMA* STRAINS IN ALBINO RATS

by

P. H. VAN THIEL

(Received December 7, 1955).

JACOBS and JONES (1950) in Bethesda (Md), using the R H strain of *Toxoplasma gondii* Nicolle and Manceaux, described that albino rats weighing about 150 grams injected by the intraperitoneal route have been found to harbour toxoplasmas as long as 7 months after infection. The rats manifested only a slight transient illness characterized by a roughened coat and apparent depression lasting for two or three days during the second week after infection. RUCHMAN and FOWLER (1951) in Cincinnati showed that the parasites were present with great regularity on the fourth day and even up to a period of two years in the brain after intraperitoneal inoculation of the same strain into albino rats weighing between 200 and 350 grams. Owing to their "protected" environment in the brain *Toxoplasma* seemed to survive in this tissue indefinitely. This observation is of particular importance, because PERRIS, BRIGHAM and PICKENS (1943) reported the occurrence of chronic toxoplasmosis in wild rats in Savannah, Georgia. It is also important because rat passages pointed to the possibility of maintaining the parasites indefinitely in the laboratory after intraperitoneal inoculation of rats instead of mouse passage which are more commonly used.

The result of the above mentioned examinations in the United States do not agree with those by CALLOT and PUECH (1952) in Strassburg performed with a Netherlands strain, probably Bijkerk. These authors could never find back toxoplasmas in the brains of adult albino rats after intraperitoneal inoculation, neither in smears nor by subinoculation. Distinct pathological manifestations could

never be observed in rats inoculated in this way, even not in young rats of 3 to 6 days old.

This quite other experience with a strain of Netherlands' origin lead me to examine 1. the behaviour of other *Toxoplasma* strains isolated from clinical cases in the Netherlands after intraperitoneal inoculation of albino rats. 2. whether intracerebral inoculation of young rats might give a better result.

Intraperitoneal inoculation was made with 1 ml 10 per cent suspension of the brains of mice that died after intracerebral inoculation with the different strains. Intracerebral inoculation was performed with 0.1 ml of the same suspension. The survival of toxoplasmas in the brains of the rats was examined by suspending the right half of the brains, where the toxoplasmas had been inoculated, in Tyrode solution and by injecting 1.0 ml of the suspension each time into two mice. When these mice survived, their whole brains were often inoculated into two other mice. Results are given in Table 1.

DISCUSSION OF THE RESULTS.

1. The Netherlands' virulent *Toxoplasma* strains van den Oever, Deelen, Colijn and Pijper did not persist in the brains of albino rats after intraperitoneal inoculation (series A and B). The result obtained by JACOBS and JONES, RUCHMAN and FOWLER could therefore not be corroborated, no more than by CALLOT and PUECH.
2. Intracerebral inoculation gives for the examined Netherlands' *Toxoplasma* strains better chance to keep them alive in the brains of rats. Only strain Colijn survived 12 months; the strains van den Oever, Deelen and Pijper could not be found back after one month after the infection (series D).
3. Series E shows that it is possible that the four above mentioned strains survive up to 25, successively 31 days in the brains. In series D strain Deelen (next to strain Colijn) survived as well for one month; the other strains did not survive.
4. The results of the series C, D and E do not completely agree, for the following reasons: *a.* In series E strain van den Oever survived for one month, not in series D. *b.* In series C a partial negative result was obtained with the strain Colijn after three days and a negative result after 4 days; in series D also a negative

TABLE 1.

Number of rats	Weight of rat in g	Way of infection	<i>Toxoplasma</i> strain	Died		Killed after	Result sub-inoculation of 1/2 rat brains on each time 2 mice	Result sub-inoculation of 1/2 mouse brains on each time 2 mice
				after	result			
Series A								
5	150	intrapertitoneal	v. d. Oever Deelen	—	—	1,2,3,4,5 months	negative	—
5	150	intrapertitoneal		—	—			
Series B								
3	80	intrapertitoneal	v. d. Oever Deelen Colijn Pijper	—	—	1,2,3 months	negative	—
3	80	intrapertitoneal		—	—			
3	80	intrapertitoneal		—	—			
3	80	intrapertitoneal		—	—			
Series C								
3	120	intracerebral	v. d. Oever Deelen Colijn Colijn Colijn	—	—	2,3,4 days	positive	—
3	120	intracerebral		—	—			
1	120	intracerebral		—	—			
1	120	intracerebral		—	—			
1	120	intracerebral		—	—			
Series D								
1	80	intracerebral	v. d. Oever v. d. Oever Deelen Deelen Colijn Colijn Colijn Pijper	4 days	+	1,3,12 months	—	negative
3	80	intracerebral						
1	80	intracerebral						
3	80	intracerebral						
1	80	intracerebral						
3	80	intracerebral						
3	80	intracerebral						
4	80	intracerebral						
Series E								
5	80	intracerebral	v. d. Oever Deelen Deelen	—	—	4,7,14,22,31 days	positive	—
3	80	intracerebral		—	—			
2	80	intracerebral		—	—			
4	80	intracerebral	Colijn Pijper Pijper	—	—	5,9,17,25 days	1 pos., 1 neg. positive ¹⁾	—
1	80	intracerebral		4 days	+			
4	80	intracerebral						

¹⁾ One mouse survived with pseudocysts in the brains.

- result after one month. This contradicts further experience with this strain, as appears from what is mentioned under 2. Intracerebral inoculation does not always succeed therefore.
5. From the results mentioned under 4 it may not be concluded that the intracerebral inoculation has not been performed well. This is apparent from experiments with strain Pijper. Out of 8 rats inoculated at the same time with this strain (series D and E) 4 were very ill, the other 4 did not show any trouble from the infection. The last mentioned group was examined after 5 to 25 days; toxoplasmas were found in the brains. Of the first mentioned group, where the infection certainly succeeded, the brains were examined after 1 to 12 months, however with a negative result. From this it is apparent that this strain did not survive longer than one month in the rat.
 6. It may be concluded that differences exist between the four examined Netherlands' strains. Assuming that no difference exists between the albino rats used in U.S.A., Strassburg and Leiden, *Toxoplasma* strain differences also exist between the American strain R H on one side and the Netherlands' strains on the other side. It may be added that our laboratory possesses a strain Burk, isolated from man, which does not kill mice after subcutaneous inoculation, but always causes a chronic infection with pseudocysts in the brains; moreover a strain isolated from a dog which after intracerebral inoculation into a mouse does not kill the mouse by an acute infection, but always forms pseudocysts. JACOBS (1953) as well observed the existence of strain differences of *Toxoplasma*. FRENKEL (1953) described characteristics of toxoplasmosis in mice by R H and C J strains.
 7. Only the strain Colijn can be maintained on the laboratory for many months by intracerebral inoculation of young some 80 grams weighing albino rats.
 8. Out of 44 intracerebrally inoculated rats only 4 died by toxoplasmosis (inoculated with strains van den Oever and Pijper, series D and E). This agrees with JACOBS (1953) who described that rats which manifest little evidence of infection following peripheral inoculation, may die after cerebral inoculation of *Toxoplasma*. The symptoms mentioned by JACOBS and JONES (1950) have not been observed in the other rats. Sometimes the rats seemed ill.

S u m m a r y.

1. The Netherlands' *Toxoplasma* strains van den Oever, Deelen, Colijn and Pijper do not persist in the brains of albino rats after intraperitoneal inoculation. The result obtained by JACOBS and JONES (1950) with an American strain could therefore not be corroborated for Netherlands' strains.
2. The strain Colijn may survive in young albino rats after intracerebral inoculation for 12 months. The other strains could not be found back in the brains after one month after the infection.
3. Strain differences exist between the examined Netherlands' *Toxoplasma* strains and between these strains and the American R H strain.
4. Only 2 out of 44 intracerebrally inoculated young rats died by toxoplasmosis.

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THE TAXONOMIC STATUS OF *TOXOPLASMA GONDII*

by

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(Received February 2, 1956).

Much controversy exists concerning the taxonomic status of *Toxoplasma gondii* Nicolle and Manceaux. GUSTAFSON, AGAR and CRAMER (1954) mention it a perplexing problem, HABEGGER (1953) "le problème le plus ancien et le plus obscur dans l'étude de la toxoplasmose".

There is general agreement that *Toxoplasma* is probably a protozoon, but little as to what kind of protozoon it may be. POISSON (1953) classified it among the *Protozoa* of uncertain affinities. CROSS (1947) judged that none of the observed characteristics of *Toxoplasma* would prevent its allocation with the *Mastigophora*. WESTPHAL (1954) put it with the *Trypanosomidae*, BRUMPT (1949), SWELLENGREBEL and IHLE (1953) with unclassified *Sporozoa*, KUDO (1950) with the *Babesiidae* (order *Haemosporidea*); WENYON (1926) classified it between the orders *Coccidiida* and *Adeleidea*, CHATTON and BLANC (1917) near the *Coccidia*, CHANDLER (1955) between *Babesiidae* and *Haemogregarinidae*, also near the *Coccidia*. KEAN and GROCOTT (1945), BIOCCA (1949), CRAIG and FAUST (1949), MANWELL and DROBECK (1953) pointed to affinities with the *Sarcosporidea*. However, there seems, according to the last mentioned authors much reason to question the general practice of including either with the *Protozoa*. WENYON (1926) as well shares this hesitation. Therefore DOFLEIN and REICHENOW (1929) classified *Toxoplasma* only with reservation among the *Protozoa*. They considered it possible that *Toxoplasma* would be one of the *Fungidae*.

Concerning these affinities a few remarks:

1. With the *Mastigophora* and *Trypanosomidae*. A flagella like filopodium in *Toxoplasma* is described by SPLENDRE (1913), CROSS (1947), PIEKARSKI (1950), HIRSCHLEROWA and KOZAR (1952) and WESTPHAL (1954). SCHMIDT-HOENSDORF and HOLZ (1953), BRINGMANN and HOLZ (1954), GUSTAFSON, AGAR and CRAMER (1954) and the present author have not observed any flagella, cilia or other obvious projections on any cell by electron microscopy. On account of their photograms BRINGMANN and HOLZ presume that flagellate like structures, observed by other authors, have been mucous threads.

Toxoplasma may be considered according to WESTPHAL (1954) a relative of *Leishmania* which, by increasing adaptation to the vertebrate host, has freed itself of a blepharoplast and, like *Trypanosoma equinum*, cannot develop any more in arthropods. Like *Trypanosoma* and *Leishmania*, *Toxoplasma* has a wide range of warm blooded hosts. According to MANWELL and DROBECK (1953) *Toxoplasma*, as well as *Sarcocystis*, would have preserved their longitudinal binary fission and their marked antero-posterior differentiation from their flagellate descent.

2. With the *Sporozoa*. WESTPHAL (1954) considers this affinity unlikely by the host specificity existing within this class. MANWELL and DROBECK (1953) added the apparent lack of any intermediate host or vector.

The disposition of *Toxoplasma* with the *Babesidae* seems illogical according to MANWELL and DROBECK (1953), since the genera of this family are parasitic in the erythrocytes of vertebrates and two of the three known to be tick-transmitted.

The affinity with the *Coccidia* is founded on the resemblance in shape of *Toxoplasma* with the merozoite of a coccidian (JACOBS, 1953). CHATTON and BLANC added the occurrence of schizogony. This is mostly doubted, but after my opinion still, although seldom (we found it in the brains of intracerebrally infected mice, also according to BRUG and Vos, 1942), occurring. Both arguments, however, are not sufficient for accepting this affinity.

3. With the *Sarcosporidea*. BIOCCA (1949), MANWELL and DROBECK (1953) showed that *Sarcocystis* and *Toxoplasma* are rather closely related, on account of their intracellular development, binary division, morphological resemblance with the spores of *Sarcocystis*, motion, almost complete lack of host specificity, lacking of an arthropod in which it passes part of its cycle and similar serology

concerning SABIN and FELDMAN dye test (MÜHLPFORDT, 1951), although this remains to be confirmed. RACCUGLIA (1952) holds the view that both genera must be considered as a specific class of micro-organisms included in the *Protozoa*, of which these genera are the most elementary representatives.

4. With the *Fungidae*. Because it has been long recognized that neither *Sarcocystis*, nor *Toxoplasma* fit well into the scheme of protozoan classification, SPINDLER and ZIMMERMAN (1945) sought for a relation with the *Fungidae*. They described having been able to observe that *Sarcocystis* develops typical fungal stages in sterile dextrose culture solution. MANWELL and DROBECK (1953), however, have been unsuccessful in growing *Toxoplasma* in this solution. CROSS failed to grow it also on SABOURAND's fungal medium. PIEKARSKI (1950) rightly remarks that this does not without more speak against the nature of a Fungus. A better argument is that CROSS observed that *Toxoplasma* reacts differently to FEULGEN's stain than do yeasts, and that *Toxoplasma*, also according to the author's own experience, is Gram negative, *Fungidae* being Gram positive (RACCUGLIA 1952; HABEGGER, 1953).

Much uncertainty exists therefore concerning the systematical status of the parasite, mainly in consequence of the fact that the morphological examination did not avail sufficient support. The use of phase contrast microscopy did not yield major advances from its application (WEINMAN, 1952). Therefore we tried to get more details by electron microscopy. A few publications on that field recently appeared.

BRINGMANN and HOLZ (1954) studying whole specimens of *Toxoplasma*, suspended in hot distilled water to gain transparency, observed a pole structure (see further on) and fibrils extending over two thirds of the cell surface taking their origin from basal granules surrounding this pole. By the fact that these fibrils cross each other, an oblique square shaped figure arises. Unfortunately the paper did not contribute to the solution of the problem of classification of *Toxoplasma*. Neither did the detailed investigation by GUSTAFSON, AGAR and CRAMER (1954). Electron micrographs of thin sections of *Toxoplasma* revealed morphological details hitherto unsuspected from study by traditional techniques. At the acute end a very distinct organelle, called conoid, some peculiar longitudinal filamentous inclusions, following a longitudinal course through the

cell, roughly cylindrical bodies of a dense homogeneous material, called toxonemes, appeared to be peculiar to *Toxoplasma*. As the toxonemes approach the conoid, they become very slender and tortuous, and can be seen to converge, entering into the base of the conoid.

OWN ELECTRON MICROSCOPE STUDIES ¹⁾.

By the use of the electron microscope we studied whole specimens of *Toxoplasma*. Peritoneal exudate of intraperitoneally *Toxoplasma* infected mice was washed in Tyrode solution, then in distilled water for half an hour at 3000 speed. The plasmatic ribonuclein acid was not removed according to the method described by BRINGMANN and HOLZ (1954) in order to damage the interior of the parasite as little as possible. Notwithstanding the thickness and the in-transparency of the parasite a few details have been observed in two specimens, worth mentioning.

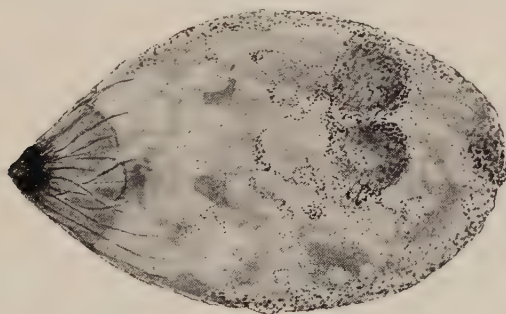


Fig. 1. Drawing from electron photo of *Toxoplasma gondii*, 12500 \times .

Specimen no 1. (Fig. 1). At the acute probably anterior pole on nearly one fifth part of length a somewhat darker part is situated which is bounded at the part directed to the blunt end by a more or less sharp one quart circle shaped line. In this part 14 very fine fibrils can be observed, which extend fan like from the dark coloured

¹⁾ The photographs are made by the late Mr. J. D. BAKKER in the Laboratory for Flowerbulb Research at Lisse.

pole to the caudal end. The middle fibrils can be discerned best. This terminal darker pole is called "conus".

Specimen no 2. (Fig. 2). At the acute end is a polar part invaginated into a collar. Further longitudinal filaments are visible.

We have not succeeded in finding back the structures, observed in both specimens, in other specimens. It does, however, not seem possible to regard them as artefacts.

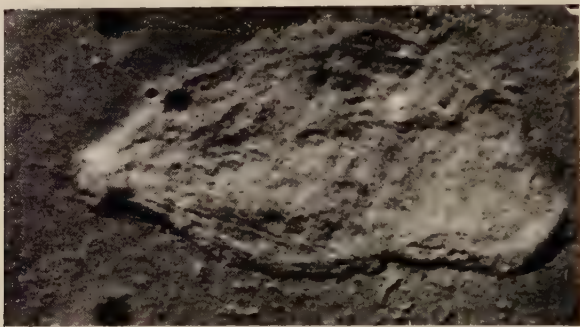


Fig. 2. Electron photo of *Toxoplasma gondii*, 11500 \times .
(Shadow-cast: Palladium).

RELATION OF THE OBSERVED STRUCTURES TO THE OBSERVATIONS BY OTHER AUTHORS.

The conus, here bounded by a light coloured line, is visible in the electron photograph 5 of *Toxoplasma* by BRINGMANN and HOLZ (1954), hardly visible as a somewhat darker part in photograph 4. The lines visible in this conus are the fibrils described by these authors. The conus as a sharply bounded part may be found also in the photos 2 and 3 in the paper by GUSTAFSON and co-workers.

In vital stained toxoplasmas and in toxoplasmas stained according to GIEMSA and KIEWIET DE JONGE frequently a pole darker coloured than the other part of the body may be observed. It is pictured also by CHATTON and BLANC (1917). After the author's opinion the conus is not identical with the conoid of GUSTAFSON and co-workers, on account of the following facts:

1. GUSTAFSON's conoid measures 0.15 to 0.25 μ in diameter and 0.2 to 0.3 μ in length. The author's conus has a length of 1.3 μ , namely one fifth of the parasite.

2. The conoid is visible in spec. 1 (Fig. 1) at the acute pole of the conus as a very dark part.

3. The conoid is probably represented also by the anterior pole of spec. 2 (Fig. 2) invaginated into the collar. According to GUSTAFSON and co-workers the conoid tends to protrude in occasional extended cells, but more often it is somewhat retracted into the cell substance. It is possible, after the author's opinion, that the conoid has a function in the perforating of the parasite into the host cell. GUSTAFSON and co-workers suggested that the conoid can be designed as a mouth structure, a penetration device or a vestige of protozoal structure such as a cytopharynx.

The conoid seems to be also identical with the sharp organelle which BRINGMANN and HOLZ observed and which they called "trombicula".

The fibrils observed by the author in the conus are very probably identical with the fibrils observed by BRINGMANN and HOLZ, because they are likewise thin and because a few of them cross each other and clearly extend beyond the rather sharp separation of the conus into the other part of the cell body. This supposition being true, the distinct line in the middle of the posterior part of the conus might be explained as a fibril which is broken and consequently situated transversally. This line, therefore, does not mean a terminal limitation of the conus.

That no more fibrils are found in specimen 1 is probably caused by the bursting of the parasite to the right in the middle of the parasite, by which part of the cytoplasm has been pushed out (this is not figured). This perhaps made the conus and the fibrils visible.

The fibrils observed by BRINGMANN and HOLZ and by the present author are very probably identical with the fine anterior ends of the toxonemes of GUSTAFSON and co-workers, but it is not possible to decide concerning their posterior parts.

THE SYSTEMATIC POSITION OF TOXOPLASMA.

On account of the observations by BRINGMANN and HOLZ, by GUSTAFSON, AGAR and CRAMER and by the present author it might be presumed that an affinity exists between *Toxoplasma* and *Gregarines* (Order *Eugregarinida* of *Sporozoa*).

In an infected *Aedes albopictus* RAY (1933) observed two types of trophozoite of the Gregarine *Lankesteria culicis* (Ross), one with a very delicate epimerite and the other with a well developed one



Fig. 3. *Lankesteria culicis* (reproduced from RAY, 1933). 1 and 2. intracellular stage in anterior portion of the mid gut of *Aedes albopictus*; 3. young stage anchored to the epithelium of the posterior portion of the mid gut; 4. adult gregarine.

(reproduced in Fig. 3). The cytoplasm of both is fine granular, except at the anterior end, where a clear cone-like zone can be distinguished from the rest. In smears and sections this clear area took up a dark, homogeneous stain, so that there is a dark zone at the anterior end. Therefore he was unable to see the fibres arranged "fanwise" which WENYON (1911), according to RAY, observed in



Fig. 4. *Lankesteria ascidia* (reproduced from DOFLEIN and REICHENOW, 1929), adult gregarine.

Lankesteria culicis. In the adult *Lankesteria ascidia*, pictured by DOFLEIN and REICHENOW (1929) in Fig. 853 and reproduced here in Fig. 4, this cone is very distinct. RAY considered this cone like area comparable with the protomerite of septate *Gregarines*. A striking similarity exists between this conelike area and my conus.

According to WENYON (1911) the epimerite is a vesicle and possibly a suction organ. This organ could be homologous with the conoid or trombicula of *Toxoplasma*. According to RAY the shape of the epimerite depends on the region of the gut the *Gregarines* inhabit. Those which infect the oesophagus and anterior part of the mid gut, where the chitinous lining is thin and smooth, are always of intracellular habitat and possess very feebly developed epimerites.

When this affinity exists indeed, *Toxoplasma* could be a parasite of invertebrate animals which lost their complicated cycle within the host by "adaptation" at vertebrate animals and which developed since then only in an intracellular way.

Summary.

The taxonomic status of *Toxoplasma gondii* is reviewed. Two electron photographs are figured, in which up to now non described structures are visible: 1. at the anterior end a conus, which is not identical with the conoid of GUSTAFSON, AGAR and CRAMER; 2. the invagination of this conoid into the anterior pole (conus).

Arguments are mentioned speaking for the possibility of a relationship of *Toxoplasma* with the *Gregarines*. So long as no more arguments are put forward, it deserves recommendation to classify *Toxoplasma* with unclassified *Sporozoa*.

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SACCHAROMYCES CAPENSIS NOV. SPEC. A NEW YEAST FROM SOUTH AFRICAN GRAPE MUST

by

J. P. VAN DER WALT and **IRMGARD T. TSCHESCHNER**

(Received February 1, 1956).

In a recent examination of the predominant yeasts associated with fermenting grape musts from the Western Cape Province, a strain was isolated which on classification appeared distinctly different from any previously described species.

The following description is based on the procedures described by LODDER and KREGER-VAN RIJ (1952) for the classification of yeasts.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C. the cells are round to oval, $(5-9) \times (7-10)\mu$, single or in pairs. After 1 month at 17°C. a sediment is formed.

Growth on malt agar: After 3 days at 25°C. the cells are round to oval, $(4.5-10) \times (5.0-12.5)\mu$, single or in pairs. After 1 month the streak culture is greyish brown, somewhat shiny, smooth with little warts. The margin shows a delicate structure.

Slide cultures: No pseudomycelium is formed.

Sporulation¹⁾: 1-4 smooth, round or slightly oval spores are formed per ascus. No conjugation was observed (Fig. 1).

Fermentation:	Glucose	+	Maltose	—
	Galactose	—	Lactose	—
	Saccharose	+	Raffinose	$\frac{1}{3}$

¹⁾ Spores were observed on malt agar and on the common sporulation media.

Sugar assimilation:	Glucose	+	Maltose	—
	Galactose	—	Lactose	—
	Saccharose	—		

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: Slight growth.

Splitting of arbutin: Absent.



Fig. 1. *Saccharomyces capensis*. Spores on Gorodkova agar after 1 week (1000 \times , sunlight dark field microscopy).

DISCUSSION.

As this budding organism is sporogenous, ferments glucose vigorously and forms no pellicle on liquid media, it must be classified as a species of the genus *Saccharomyces* (Meyen) Reess.

LODDER and KREGER-VAN RIJ (1952) recognise two species which ferment glucose, saccharose and raffinose for a third part and which also assimilate only these sugars, *viz.* *Saccharomyces rosei* Guilliermond (Lodder et Kreger-van Rij) and *Debaromyces globosus* Klöcker. Despite the similarity in its behaviour towards the number of carbohydrates examined, the strain from must shows definite morphological differences from *Sacch. rosei* and *Deb. globosus*.

It differs from *Sacch. rosei* in the first instance in that its cells are larger. The round mature cells of *Sacch. rosei* measure only $(2.0-6.0) \times (2.0-6.9) \mu$ in malt extract, while the round to oval cells of the strain under discussion measure $(5.0-9.0) \times (7.0-10.0) \mu$ under the same conditions. In the second instance all sporulating strains of *Sacch. rosei* form protuberances resembling conjugation tubes, during sporulation. This feature, quite characteristic for

Sacch. rosei is, however, completely lacking in the strain from must.

The differences which the strain shows from *Deb. globosus*, on the other hand, are as distinct. In the first instance there is again a difference in cell size. KLÖCKER (1909) gives the cell measurements for *Deb. globosus* as $(4.5-5) \mu$ in malt extract and accordingly much smaller than those observed for our strain. In the second instance *Deb. globosus* is characterised by the fact that its ascospores have a distinctly warty cell wall – a feature which is absent in the strain under discussion. Furthermore, GUILLIERMOND (1912) illustrates the formation of protuberances resembling conjugation tubes during the sporulation of *Deb. globosus* as well.

The differences which the yeast from must shows from either *Sacch. rosei* or *Deb. globosus* are sufficient to establish it as a new species. The name *Saccharomyces capensis* is proposed, naming it for the Cape Province from where it was isolated.

A sub-culture of *Sacch. capensis* has been deposited in the Yeast Collection of the Centraal Bureau voor Schimmelcultures in Delft.

Saccharomyces capensis nov. spec.

In musto maltato cellulae rotundae aut ovidiae $(5.0-9.0) \times (7-10) \mu$ singulae aut binae. Sedimentum formatur.

In agar maltato cellulae rotundae aut ovidiae $(4.5-10) \times (5.0-12.5) \mu$ singulae aut binae.

Cultura (post unum mensem, 17°C.) griseola flavifusca, parum nitens, glabra cum verrucis parvis, in margine structura subtilis.

Pseudomycelium nullum.

Ascosporae glabrae, rotundae aut sub-ovidiae, 1–4 in asco.

Fermentatio glucosi, sacchari et raffinosi pro tertia parte. In medio minerali cum glucoso et saccharo crescit. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico parum crescit. Arbutinum non finditur.

A c k n o w l e d g e m e n t s

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(From the National Institute for Public Health, Utrecht, and the Laboratory of the Municipal Slaughterhouse, Deventer).

INFECTION IN PIGS WITH A RARE STRAIN OF *S. CHOLERAE SUIIS*

by

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(Received February 1, 1956).

It is well-known to any pathologist and bacteriologist as well as to any veterinary practitioner in this country that swine-plague has shown other aspects in post-war years than in former decades. This change concerns in the first place the pathological-anatomical differences, but besides, the different bacteriological findings. Although formerly in addition to the primary virus, *S. cholerae suis* was found as a secondary infection in a great number of cases, this germ was, as far as we know cultivated in post-war years only very rarely from animals slaughtered on account of swine-plague. The under-mentioned case shows again that the exception proves the rule and is suitable at the same time to draw attention to the fact that also the present form of swine-plague may be attended with Salmonellosis. On August 12, 1955 a herd of 37 pigs were conveyed under police-escort to the Public Slaughterhouse at Deventer. They came from a farm where the Inspector of the Veterinary Service had confirmed swine-plague. 6 Pigs were ill; they were marked especially by the very pale colour of the skin. Not much attention was paid to the inspection before slaughtering, that is to say the temperature was not taken, because the diagnosis swine-plague was already definitely established. In the animals slaughtered the following lesions were observed:

The lgl. bronchiales and submaxillares were swollen and had a red-marbled aspect. Pneumonia was present in all 6 animals. One pig had an affection of the intestines *viz.*, round foci of inflammation, the size of a few millimetres, resembling „boutons”. The livers all contained a great many parasitic nodules. The lgl. mesenteriales

were swollen in a few animals, but not red-marbled. The kidneys were swarmed with petechia, which also occurred in the lungs of several pigs and in one animal under the costal pleurae. The sick pigs that had been slaughtered were of a much paler colour than the other animals of the herd. Before having the pigs sterilized a bacteriological examination was made, in view of possible secondary infection. For this reason material from the spleen was cultured on agar medium. After 18 hours incubation in 5 out of the 6 sick pigs abundant growth of highly motile, Gram negative rods was observed. Agglutination reactions with polyvalent *Salmonella* O-serum and with the specific H-serum of *S. cholerae suis* were positive. Brilliant green - phenol red-plates were inoculated from one colony and after incubation they were sent to the National Salmonella Centre, where by serological examination the antigenic formula VI, VII : c : 1,5 was established. *S. cholerae suis*, *S. paratyphi* C and *S. typhi* *suis* have this formula in common. These types can be distinguished biochemically with the aid of arabinose and trehalose, which sugars are not fermented by *S. cholerae suis*, whereas they are by the other 2 types. The strains cultivated from the 5 sick pigs were all arabinose- and trehalose-negative.

According to literature (KAUFFMANN, 1954) it could be expected that these diphasic strains would not form H_2S . It appeared, however, that all these strains developed H_2S in sulfite agar within 24 hours. As is well known *S. cholerae suis* (formerly known as *S. suispestifer* or *S. suispestifer* var. *Kunzendorf*) is divided into 2 groups, viz., the American strains, which are diphasic and consequently possess the H-antigens c and 1,5 and do not form H_2S , and the European strains (*S. cholerae suis* var. *Kunzendorf*), which are monophasic and only possess the non-specific 1,5-antigen and form H_2S .

Though finding *S. cholerae suis* was already an exception - in this country the strain was isolated in pigs only once in the past 8 years - it was even more remarkable that the germ did not behave biochemically and serologically as the representatives of the two groups mentioned. Such strains had, although very rarely, been described before, as appears from the undermentioned survey, compiled by BRUNER and EDWARDS (1939).

From this survey may be seen that the diphasic, H_2S positive form is only found in the pig and that even rarely.

Species of Animals	Number	H ₂ S	H-antigen
pig	10	—	diphasic
	111	+	monophasic unspecific
	3	+	diphasic
fox	2	—	diphasic
	15	+	monophasic unspecific
cattle	1	—	diphasic
	1	+	monophasic unspecific
dog	1	+	monophasic unspecific
chicken	1	—	diphasic
canary	1	+	monophasic unspecific
man	1	—	diphasic
	3	+	monophasic unspecific

In order to check if any other forms were present in our cultures 100 separate colonies were examined; they all appeared to be diphasic and H₂S-positive.

Since according to KAUFFMANN forming H₂S should be a more important characteristic with the classification mentioned than the antigenic formula, it is clear that we have to deal here with a European, diphasic strain of *S. cholerae suis* var. *Kunzendorff*.

Unfortunately it is impossible to find out which *S. cholerae suis*-strains formerly occurred in this country in animals suffering from swine-plague, as the differential-diagnosis had not yet reached such an advanced stage and the strains have not been kept. In the course of time changes may set in which are of epidemiological interest; this appears, among other things, from the fact that in America since 1939 no longer the American type has occurred, as was the case before that time, but almost exclusively the European type (SAPHRA and WASSERMANN, 1954). In this connection it is interesting to mention that this germ, which was formerly sometimes looked upon as non-pathogenic for man, takes at present a very important place in America as a pathogenic germ. *S. cholerae suis* was found in man only twice in Holland in the past 8 years, whereas SAPHRA and WASSERMAN (Salmonella Centre, New York) could isolate this germ in the past 14 years 329 times from material originating from man.

Even more important than these absolute figures, which can

hardly be converted per number of inhabitants – as in America also strains from many Central American States are determined and because there are several Salmonella-Centres in the U.S. – is the fact that out of 4000 strains sent in and originating from man, 7.2% appeared to be *S. cholerae suis*. Owing to this *S. cholerae suis* takes the 5th place, after *S. typhi murium*, *S. newport*, *S. oranienburg* and *S. montevideo*. In this country *S. cholerae suis* was found only twice among 2523 strains originating from man (0.08%).

It is an established fact that in America the pig must be regarded as a primary reservoir for *S. cholerae suis*, which clearly finds expression through examinations of pork and of meats prepared from pork.

Summary.

In 5 animals out of a herd of pigs slaughtered on account of swine-plague *S. cholerae suis* was isolated as a secondary infection. These strains appeared to differ from the diphasic *S. cholerae suis*. Unlike these they were H₂S-positive.

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KLUYVEROMYCES — A NEW YEAST GENUS OF THE *ENDOMYCETALES*

by

J. P. VAN DER WALT

(Received March 7, 1956).

In a recent survey of the yeast flora associated with surface soils, a very remarkable budding species was isolated from a sample of uncultivated earth. It possessed both an oxidative and a strong fermentative dissimilation. The most striking feature of this organism, however, was the formation, generally preceded by conjugation, of an unusually large type of cell. On reaching maturity these cells often measured up to $(25 \times 29) \mu$. Their contents, at first granular, were eventually transformed into regular long oval to reniform bodies. The number of these per cell varied considerably. In many instances more than 50 per cell could be counted. In other cases it was impossible even to establish their exact number. These bodies were acid-fast and stained selectively with the Schaeffer-Fulton malachite green-safranin stain and Mann's methyl blue-eosin stain (Figs. 1, 2 and 3). Moreover, these bodies proved to be more heat resistant than the vegetative cells. Cultures of vegetative cells were inactivated when subjected to a temperature of 70°C . in the absence of moisture for 10 minutes, while cultures containing these endogenous, acidoresistant-acidophilic bodies survived this treatment.

The endogenous origin, the acidoresistant-acidophilic character and heat resistance of these bodies left very little doubt as to their true ascosporeogenous nature and the ascogenous character of the yeast.

EXPERIMENTAL METHODS AND RESULTS.

The following description is based on the application of the

standard methods of LODDER and KREGER-VAN RIJ (1952) for the identification of yeasts.

Growth in malt extract: After 3 days at 25°C. the cells are short oval to oval $(2.5 - 10) \times (3.1 - 13.8) \mu$, single or in pairs (Fig. 4). Conjugating cells and zygotes measuring up to $(25 \times 28) \mu$ are present (Figs. 5, 6 and 7). After 1 month at 17°C. a pellicle and sediment are formed.

Growth on malt agar: After 3 days at 25°C. two types of cells are formed. They are either oval $(2.5 - 5.0) \times (3.1 - 6.3) \mu$ or short oval $(5 - 6.8) \times (6.3 - 8.7) \mu$, single or in pairs. Conjugating cells and large zygotes are also present. After 1 month at 17°C. the streak culture is yellowish-brown, somewhat shiny.

Slide cultures: A pseudomycelium of the 'Mycocandida'-type is formed.

Sporulation¹⁾: An isogamous or heterogamous conjugation usually precedes ascus formation. The zygote increases greatly in size. Its cell wall thickens and the contents become granular. The granular substance is transformed into ascospores. The ripe asci may measure up to $(25 \times 29) \mu$. The asci are multispored. The spores are smooth, long oval to reniform. Asci may also be formed without preceding conjugation.

Fermentation:	glucose	+	maltose	—
	galactose	+	lactose	—
	saccharose	+	raffinose	$\frac{1}{2}$
Sugar assimilation:	glucose	+	maltose	—
	galactose	+	lactose	—
	saccharose	+		

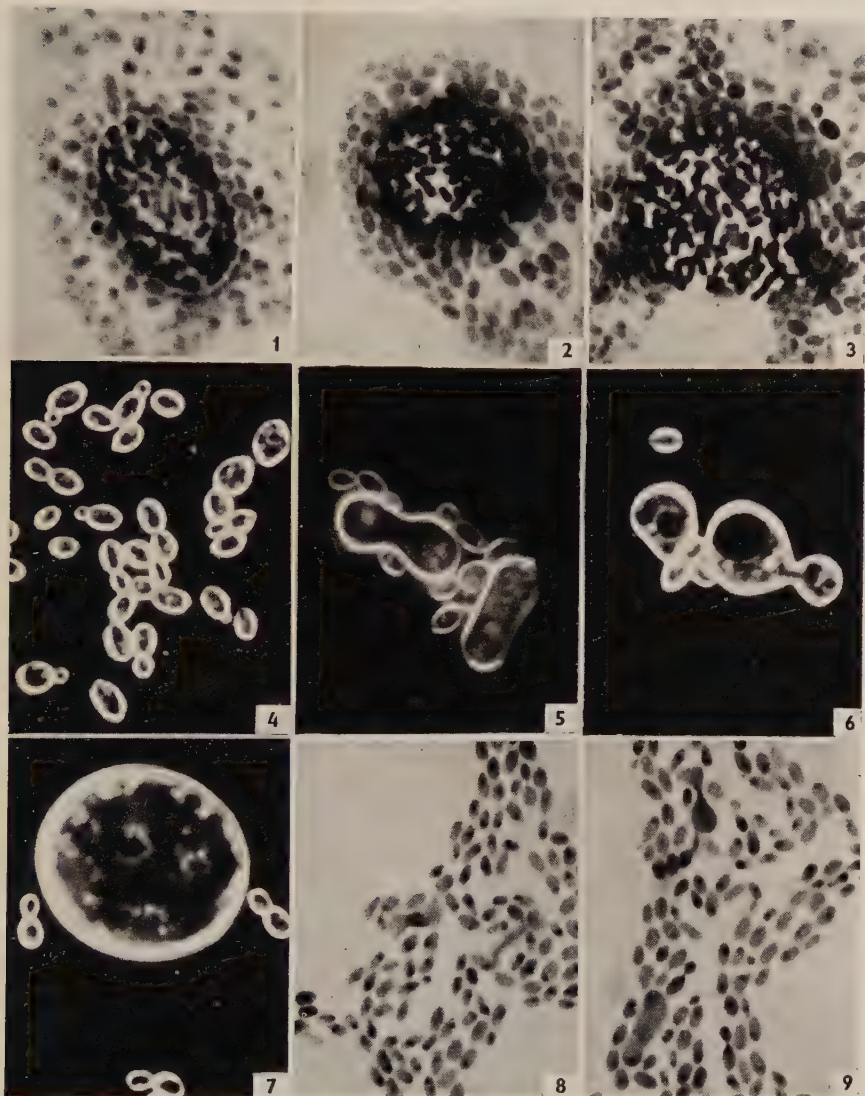
Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: Slight growth.

Splitting of arbutin: Absent.

Cytological examination: For nuclear staining, Heidenhain's haematoxylin stain was employed as recommended by GUILLIERMOND (1912). Suspensions were prepared from an 18 hr. malt agar culture. Slides were fixed in Bouin's picroformol for 12 hr., mordanted for 12 hr. in a 2.5 per cent solution of ferric alum, washed and stained with a 1 per cent solution of haematoxylin for 24 hr. The stained slides were differentiated in 2.5 per cent ferric

¹⁾ Sporulation was observed on malt agar and on Gorodkowa agar.



Figs. 1, 2 and 3. *Kluyveromyces polysporus*. Multispored asci on Gorodkova agar after 7 days. Stained with Schaeffer-Fulton spore stain ($\times 1,000$).

Fig. 4. *Kluyveromyces polysporus*. After 3 days in malt extract ($\times 1,000$).

Fig. 5. *Kluyveromyces polysporus*. Isogamous conjugation in malt extract after 3 days ($\times 1,000$).

Fig. 6. *Kluyveromyces polysporus*. Heterogamous conjugation in malt extract after 3 days ($\times 1,000$).

Fig. 7. *Kluyveromyces polysporus*. Giant zygote in malt extract after 3 days ($\times 1,000$).

Figs. 8 and 9. *Kluyveromyces polysporus*. An 18-hour malt agar culture stained with Heidenhain's haematoxylin ($\times 1,000$).

alum solution. Results obtained by this method showed the cells to be either uni- or binucleate at this stage of their development (Figs. 8 and 9).

DISCUSSION.

Since the organism forms no ascogenous hyphae or ascocarps but individual asci, it must be brought to the sub-class of the *Protascales* of the *Ascomycetes*. Since its vegetative cells are uninucleate it must be assigned to the order of the *Endomycetales*.

The further classification of this species within the order will depend on what significance may be attached to its spore number. In the case of this organism it has not been possible to arrive at any constant maximum number of spores per ascus. As stated, many asci contained more than fifty spores per ascus but in isolated instances even seventy odd could be counted per ascus with some degree of certainty. Despite the great number of asci examined, there could in no case be established a constant maximum corresponding to any value of 2^n . The organism must therefore be considered as multispored. This would be in accordance with the opinion of MARTIN (1954) who considers any species of the *Endomycetales* with more than sixteen spored per ascus as multispored.

GUILLIERMOND (1912, 1928), STELLING-DEKKER (1931), SKINNER (1947) and LODDER and KREGER-VAN RIJ (1952) have all included the ascosporogenous yeasts in the single family of the *Endomycetaceae*, irrespective of the number of ascospores formed. However, it does not seem feasible to include any multispored species in any of the hitherto described genera characterised by a strong fermentative dissimilation, especially if it is borne in mind that GÄUMANN (1949) employed the formation of multispored asci not merely for generic separation, but separates the family of the *Dipodascaceae* on this basis. Likewise MARTIN (1954) separates the families *Ascoideaceae* and *Pericystaceae* on the basis of the formation of many-spored asci.

Thus the presence of multispored asci in a budding fermentative yeast would then certainly vindicate classifying this yeast at least in a separate genus. For this genus the name *Kluyveromyces* is proposed, naming the genus for the late Prof. dr A. J. KLUYVER, the President of the Centraal Bureau voor Schimmelcultures and Director of its Yeast Division. It was only a few months before his death when the revered teacher graciously consented to have his

name to this new genus. For the species the name *Kluyveromyces polysporus* is proposed.

Diagnosis of the genus *Kluyveromyces* nov. gen.

Cells oval to long oval. Vegetative reproduction by multilateral budding. A pseudomycelium may be formed.

An isogamous or heterogamous conjugation generally precedes ascus formation. Asci may also be formed without immediately preceding conjugation. The large asci are multispored.

In liquid media a pellicle may be formed.

Fermentation. Nitrate not assimilated.

A phylogenetic relationship for the genus *Kluyveromyces* must naturally be sought with the other multispored genera of which the most obvious would be *Ashbya* and *Dipodascus*. Evidence thus far obtained does not, however, favour a relationship with the first mentioned genus since its spores and also those of the parasitic yeast forms derived from it, viz., *Monosporella*, *Nematospora* and *Coccidiascus* are all characterised by a more or less curved needle to spindle shape of their ascospores – a feature which the spores of *Kluyveromyces polysporus* lacks completely.

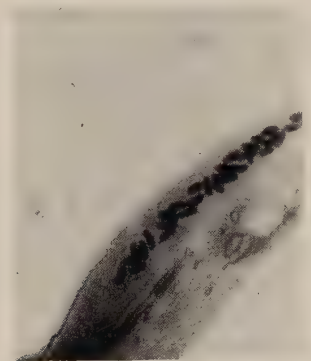


Fig. 10. *Dipodascus uninucleatus*. Long oval to reniform ascospores in a 2-week malt agar culture. Stained with the Schaeffer-Fulton spore stain ($\times 1,000$).

On the other hand, a comparison of the spores of *Dipodascus uninucleatus* Biggs (Fig. 10) with those of *Kluyveromyces polysporus* shows a marked similarity in that in both cases they are long oval

to slightly reniform. Closer comparison of the biochemical and morphological properties of these two organisms is interesting. *Dip. uninucleatus* possesses a strictly oxidative metabolism. Its growth is mould-like, the septated hyphae breaking up into oidia. The multispored asci arise either after a heterogamous copulation or parthenogenetically. *Kluyveromyces polysporus*, on the other hand, possesses both an oxidative as well as fermentative dissimilation. Its vegetative reproduction is by bud formation while its mould-like tendencies have been reduced to the production of only a pseudomycelium. Its multispored asci, however, arise not only after a heterogamous, but also after isogamous, conjugation apart from asci being formed without immediately preceding conjugation. The relationship which *Dip. uninucleatus* now shows to *Kluyveromyces polysporus* is almost identical to that which GUILLIERMOND (1909, 1912) showed to exist between *Eremascus fertilis* Stopp. and the budding ascosporeogenous yeasts. As the *Zygosaccharomyces* species may be derived from *Eremascus fertilis* via the hypothetical intermediate *Endomyces* a, it is similarly possible to conceive a parallel phylogenesis for *Kluyveromyces polysporus* by the postulation of an analogous intermediate between *Dipodascus uninucleatus* and *Kluyveromyces polysporus*. This intermediate, like *Endomyces* a, would possess the essential characteristics of both mould and yeast. Firstly, it would have to show, besides the formation of a true mycelium, a tendency towards blastospore formation. Secondly, its multispored asci, with their long oval to reniform ascospores, would have to arise not only after heterogamous, but also after isogamous, copulation.

The evolution of *Kluyveromyces polysporus* from *Dip. uninucleatus* is therefore characterised on the one hand by the retrogression of mycelium formation and gametangial sexuality and on the other hand marked by the development of blastospore formation and a fermentative dissimilation.

The reduction of the gametangial sexuality to pseudogamy, the blastospore formation and marked fermentative dissimilation in the case of *Kluyveromyces polysporus* favours placing this genus in the *Endomycetaceae* (*in sensu* Lodder et Kreger-van Rij). Its multisporeogenous character establishes, on the other hand, its obviously close relationship with *Dipodascus uninucleatus* and the family of the *Dipodascaceae*.

Latin diagnosis of the genus *Kluyveromyces* and species *Kluyveromyces polysporus*.

Kluyveromyces nov. gen.

Cellulae ovidiae. In propagatione vegetativa gemmae ab omni latere formantur: fortasse etiam pseudomycelium.

Asci cellulae aequae aut inaequae conjugatae formantur. Etiam asci sine conjugatis. Multispori asci magni. Ascosporae longovidae aut reniformes.

In mediis liquidiis fortasse pellicula formatur.

Fermentatio. Nitras kalicus non assimilatur.

Kluyveromyces polysporus nov. spec.

In musto maltato cellulae subovidae aut ovidiae $(2.5 - 10.0) \times (3.1 - 13.8) \mu$, singulae aut binae. Cellulae conjugatae praesentes proprie $(25 \times 28) \mu$ metiuntur. Pellicula et sedimentum formantur.

Formarum duarum cellulae in agaro maltato, ovidiae $(2.5 - 5) \times (3.1 - 6.3) \mu$ aut subovidae $(5.0 - 6.8) \times (6.3 - 8.7) \mu$, singulae aut binae, etiam cellulae conjugatae magnae. Cultura (post unum mensem, 17°C .) flavi-fusca, parum nitens, prope glabra. Pseudomycelium formatur.

Asci formantur hoc modo: Cellulae aequae inaequaeque conjugantur quae in magnitudine augentur. Cellulae membranum densatur. Primo materia in asco granosa est, deinde in ascosporas multas convertitur. Asci maturi metiuntur ad $(25 \times 29) \mu$. Ascosporae glabrae, longovidae aut reniformes.

Fermentatio glucosi, galactosi, sacchari et raffinosi pro tertia parte. In medio minerali cum glucoso, galactoso et saccharo, crescit. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico parum crescit. Arbutinum non finditur.

A c k n o w l e d g e m e n t.

The author wishes to thank Professor A. PIJPER for his much appreciated assistance with the photography.

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S u m m a r y.

A new budding yeast has been isolated from soil. Its most striking

feature is the formation, generally preceded by isogamous or heterogamous conjugation, of unusually large multispored asci. The organism possesses a strong fermentative ability, fermenting glucose, galactose, saccharose and raffinose for one-third. Nitrate is not assimilated. It forms a pellicle in malt extract. A pseudomycelium is also formed. Cytological examination showed the vegetative cells to be uninucleate. The relationship which this yeast shows with the *Dipodascaceae* and, in particular, with *Dipodascus uninucleatus* Biggs, is discussed. For the classification of the yeast the new genus *Kluyveromyces* was created. For the species the name *Kluyveromyces polysporus* is proposed. The Latin diagnosis of both the genus and the species is given.

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(Provincial Institute for Hygiene, Antwerp).

INFLUENCE IN VITRO OF PANTHENOL ON THE RESISTANCE OF STAPHYLOCOCCI TO ANTI- BIOTICS AND SULFONAMIDES

by

E. TRITSMANS and F. VANBENEDEN

(Received January 8, 1956).

The resistance of a very large percentage of staphylococcal infections to treatment with penicillin or sulfonamides is well known (1, 2, 3, 4).

The "broad-spectrum" antibiotics are not as useful as it was hoped, because also resistance to these antibiotics has developed. Actually, more and more cases of fulminating enterocolitis caused by drug-resistant staphylococci complicating broad-spectrum antibiotic therapy are reported (5).

Therefore, a drug abolishing or decreasing the resistance of the staphylococci would be useful, especially with penicillin, the best, least dangerous, and, unfortunately least effective drug.

In consequence, a paper of STRAHM and WETZEL (6) seemed to us very important. They report to have cured a number of infections caused by staphylococci, which were resistant to a given drug, by giving the same drug along with panthenol. Panthenol is the γ -oxyprolamine of α, γ -dioxo- β, β -dimethylbutyric acid which changes very easily in the organism into panthothenic acid, or β -alanine of α, γ -dioxo- β, β -dimethylbutyric acid (7).

Panthothenic acid is a vitamin of the B complex; panthenol, however, is used in clinic for its chemical properties. Simultaneous administration of panthenol would, according to STRAHM, markedly diminish the resistance of staphylococci to penicillin, other antibiotics, and even sulfonamides.

STRAHM and WETZEL did not rely on their clinical experience, they performed a number of bacteriological experiments in order to investigate the influence of panthenol on the resistance of sta-

phylococci in vitro. They tested the sensitivity of their strains to panthenol alone, to panthenol + penicillin, to a sulfonamide (Gantrisin) and to Gantrisin — panthenol. They used a diffusion method: the "hole test". A routine plate is inoculated with the examined strain; in each hole made in the medium a solution of an antibiotic is poured. The diameter of the inhibition zone is measured. Their results may be summarized as follows: 69.5% of their strains, resistant to penicillin alone, turned out to be sensitive to the combination of penicillin and panthenol; 77% of the sulfonamide-resistant, sensitive to sulfonamide + panthenol.

It is well-recognized that the diffusion method gives only qualitative results. The diameter of the inhibition zone not only depends on the sensitivity of the germ, but also on several other factors. This is the reason why we repeated the bacteriological investigations of STRAHM, using the reliable dilution method, in order to investigate by quantitative tests the action of panthenol. As our results are quite different from those of STRAHM, we thought it of interest to publish them.

MATERIALS AND METHODS.

Two varieties of the dilution method were used. At the beginning series of tubes with ordinary broth were used, each tube containing a determined concentration of drug with or without panthenol. All these tests were repeated with plates. As the results were entirely the same, the first results are not given separately.

Dilution method with plates.

Each plate contained a determined dilution of a drug. Series of plates were made with increasing dilutions. Each plate was then inoculated with ten different strains. The medium was 10 ml ordinary agar, to which 1 ml broth containing the drug was added; the inoculation was done with a loopful of an overnight broth culture of the strain investigated.

We used: pure penicillin; pure chlortetracycline; a mixture of equal parts of streptomycin and dihydrostreptomycin (Ambistryne). As sulfonamide, the highly soluble Gantrisin, in ampoules containing 400 mg sulfonamide and 157 mg diethanolamin pro ml. As panthenol, ampoules containing 25% panthenol in water¹⁾.

¹⁾ We wish to express our appreciation to "Produits Roche N.V., Brussel" for generous samples of panthenol (Bepantheme). We stress that the ampoules did not contain Nigapin-Nipasol as a preservative.

Origin of the strains.

Fifty strains were collected from different laboratories of Antwerp, the major part from the Provincial Institute for Hygiene itself. All were isolated in the first half of 1955 from pathological products, most of them from furuncles or pus, some from urine, conjunctivitis, etc. For the experiments only the coagulase-positive were used. These were 46; 5 of them were of the albus-type, the rest of the aureus variety. They were kept on ordinary gelose.

RESULTS.

Panthenol alone.

STRAHM noticed that even panthenol alone inhibits in some degree the growth of the germs. Then, first of all, we tried to determine the Minimal Inhibitory Concentration (M.I.C.) of panthenol, that is the greatest dilution which inhibits completely the growth.

Of 46 tested strains 36 grew slightly on a plate containing 10 ml gelose plus 4 ml 25% panthenol solution; this means 1 g panthenol in 14 ml, or 7%. In higher concentrations of panthenol the medium is even more diluted by the watery panthenol solution; therefore they were estimated unreliable. The experiments were continued with some plates containing pure panthenol. All the twelve tested strains grew on the 8% panthenol plates; eleven on the 9% plates; and six even on the 12% ones. Experiments with concentrations higher than 12% were not performed.

Penicillin alone.

For practical purpose the sensitivity of each strain was first determined. The strain was inoculated on two plates containing 0.5 U and 50 U penicillin pro ml. So the strains were divided in three groups:

Highly resistant strains: grow in 50 U pro ml.

Moderately resistant: grow in 0.5 U, but not in 50 U.

Sensitive: grow not in 0.5 U.

Penicillin with panthenol.

In order to test the influence of panthenol to the resistance of the staphylococci, 1% panthenol was added to each of a series of plates containing decreasing amounts of penicillin. A second series of plates contained the same dilutions of penicillin; instead of 1%

panthenol (this was 0.4 ml of the 25% aqueous solution) 0.4 ml physiologic aqueous solution was added.

1. Highly resistant strains.

The strain is indicated by a number. The P near the number indicates the result of the same strain on the plates to which 1% panthenol has been added. On the ordinate, penicillin, units pro ml. + indicates growth.

3200											
1600							+				
800	+	+					+			+	
400	++	+	+	+	+	+	++	+	+	+	+
200	++	++	+	+	++	+	++	+	++	++	++
100	++	++	++	++	++	++	++	++	++	++	++
	6 P	8 P	12 P	21 P	22 P	28 P	30 P	31 P	32 P	34 P	

3200											
1600											
800						+	+				
400	+	+	+		+	+	++	-			
200	++	++	++	++	++	++	++	+	+		
100	++	++	++	++	++	++	++	++	++	++	
	38 P	39 P	42 P	43 P	44 P	46 P	47 P	50 P	51 P		

2. Moderately resistant strains.

24	+										
12	+	+	+	+	+					+	
6	++	+	+	+	+	+	+				
3	++	++	++	++	++	+	+	+	+	++	
1,5	++	++	++	++	++	++	++	++	++	++	
0,75	++	++	++	++	++	++	++	++	++	++	
	7 P	25 P	29 P	36 P	37 P	41 P	48 P	52 P	55 P		

Sulfonamides.

The influence of panthenol was tested in the same manner as for penicillin. All the strains tested grew on plates containing considerable doses of Gantrisin. It is indeed well known that the medium contains a high concentration of sulfonamide-antagonists.

Sulfonamide, mg pro ml.

30

15

7,5

3,75

1,8

0,9

2 P	3 P	4 P	5 P	6 P	7 P	8 P	9 P	10 P	12 P	15 P	18 P
-----	-----	-----	-----	-----	-----	-----	-----	------	------	------	------

30

15

7,5

3,75

1,8

0,9

19 P	20 P	21 P	22 P	23 P	24 P	25 P	26 P	27 P	28 P	29 P
------	------	------	------	------	------	------	------	------	------	------

30

15

7,5

3,75

1,8

0,9

30 P	31 P	32 P	33 P	34 P	36 P	37 P	38 P	39 P	41 P	42 P
------	------	------	------	------	------	------	------	------	------	------

30

15

7,5

3,75

1,8

0,9

43 P	44 P	45 P	46 P	47 P	48 P	49 P	50 P	51 P	52 P	53 P	55 P
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STRAHM stated that succesful clinical results were obtained by adding panthenol to streptomycin and aureomycin. We decided to perform some bacteriological tests.

Streptomycin.

The strains were inoculated on plates containing 10 μ g Ambistryne pro ml. On these, 7 strains out of 46 would grow. The combination streptomycin-panthenol was tested in the usual manner.

Streptomycin, μg pro ml.

1000				+			
500	+			+		+	+
250	+	+		++	++	++	++
125	++	++	— —	++	++	++	++
	8 P	21 P	43 P	44 P	47 P	50 P	57 P

Chlortetracycline (Aureomycin).

The plates contained 10 μg pro ml. Only two strains were resistant. As it was not worth while to make a whole double series of plates, only one plate was made containing 10 μg chlortetracycline and 1% panthenol, the control plate containing only 10 μg chlortetracycline. The strains grew less profusely on the panthenol plate than on the control.

The effect of panthenol was about the same as the effect under similar conditions in the combinations with other drugs. Therefore almost the same effect on the sensitivity as in the other panthenol-combinations may be expected.

DISCUSSION.

Our results confirm the bacteriostatic proprieties of the panthenol. The M.I.C. (Minimal Inhibitory Concentration) amounting to over 10% is very high.

The panthenol-antibiotic or panthenol-sulfonamide combination inhibits better than the antibiotic or sulfonamide alone the growth of the staphylococci although we only used 1% panthenol.

It appears that the panthenol has an effect on the action of the antibiotics and the sulfonamides. This effect is constant but not very pronounced.

Contrarily to STRAHM and WETZEL we have not been able to obtain in vitro a resistant strain having become sensitive.

For strains very resistant to penicillin the presence of panthenol does not prevent staphylococci to grow in a medium containing 100 U of penicillin pro ml. As to "intermediary" strains, only three (No 41, 48 and 52) have become sensitive to three U of penicillin pro ml. We may however consider the strains as still resistant because it is admitted that a sensitive strain is inhibited by 0.5 U of penicillin pro ml, maximum 1 U pro ml.

The effect of the panthenol on the sulfonamides appears to be

more important than on the penicillin, although here also we have not been able to obtain a resistant strain having become sensitive. This superior effect on the sulfonamides seems however to be illusory to us and easy to explain.

By determining the M.I.C. of the sulfonamides alone one does not obtain a well defined limit. Below the dilution of the sulfonamides that inhibits completely the growth of the strain (f.i. to strain No 4 it is a matter of 30 mg/ml) the growth remains slight.

For the strain No 4, the growth is always slight for 1.8 mg/ml. Hence it may be expected that the combination panthenol-sulfonamides inhibits that slight growth and that the M.I.C. for the strain No 4 falls to 1.8 mg/ml.

For the penicillin this limit is more pronounced. If the M.I.C. of a strain is for instance situated at 400 U, the growth at 100 U is always considerable and the effect of the panthenol is insufficient to inhibit the growth.

Nevertheless in vitro the panthenol has a stimulating effect on the action of the antibiotics and the sulfonamides but this effect is insufficient to render a resistant strain sensitive.

The clinical results obtained by STRAHM and WETZEL find a difficult explanation in the light of our experiments. Although the presence of 1% panthenol activates the action of the sulfonamides or the antibiotics, it is probable that this concentration is not reached in vivo.

It is however impossible to conclude from what happens in vitro to what may happen in vivo. Furthermore the panthenol is easily transformed into pantothenic acid in the organism. It is possible that the "stimulating" action of the pantothenic acid is more important than that of the panthenol. We intend to investigate this in further experiments.

S u m m a r y.

The action in vitro of the panthenol on the resistance of the staphylococci to the antibiotics and sulfonamides has been studied. We have been able to establish that the panthenol has a "stimulating" effect on the action of the antibiotics and the sulfonamides. This effect was however insufficient to render a resistant strain sensitive.

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(National Institute of Public Health, Utrecht).

THE RELATIONSHIP OF Vi TYPES E₃ AND E₄ OF *SALMONELLA TYPHI* TO Vi TYPES E₁ AND D₁

by

R. TH. SCHOLTENS

(Received March 15, 1956).

CRAIGIE and YEN (1938) were able to obtain a varietal subdivision of *Salmonella typhi*, by means of the property of a given Vi bacteriophage, the Vi phage II, to develop a specific affinity for strains of the type on which it is regenerated. As a rule, this "adaptation" lysed only a certain group of strains, the homologous type, and, conversely, strains of the homologous type were attacked only by the homologous preparations.

However, CRAIGIE and YEN immediately encountered exceptions. An adaption of Vi phage II attacked Type E₁ in addition to the homologous strains. These strains, however, were not attacked by the preparation adapted to Type E₁. The newly identified type, because of this relationship to the Type E₁, was called Type E₂. Later on other types were found which bore a similar relationship to Type E₁ and were therefore called Types E₃, E₄, E₅, E₆, E₇ and E₈. Another group of related types is formed by Type D₁ with the Types D₂, D₄ and D₆.

FELIX and ANDERSON (1951) and ANDERSON and FELIX (1953) demonstrated a relation between the phage type of typhoid bacteria and the presence of natural phages in the bacteria. In the strains of given types certain natural phages were identified; when these natural phages are brought to strains of other types, cultures can be isolated from them after incubation for some time, that produce the natural phage, and are of a different type. Thus, by lysogenisation of Type A with the natural phage 29' cultures arise that react like Type 29; by lysogenisation of Type E₁ with the natural phage 29' cultures arise which, with the method of CRAIGIE and FELIX, react as Type E₇.

A good explanation of all these phenomena is that the specific resistance of the phage types is often complex and built up of the specific resistances that occur in other phage types alone, and that Vi phage II must possess virulences corresponding with each of these resistances to be able to lyse such types (ANDERSON, 1955). An example will easily illustrate this. The adaptations of Vi phage II A to the Type E_7 attack not only Type E_7 but also Types E_1 and 29. The adaptations to the Types E_1 and 29 respectively do not attack the type E_7 , however. It may therefore be assumed that the specific resistance of the Type E_7 is composed of the specific resistances of E_1 and 29, and that the Vi phage II must have acquired affinities corresponding to each of these two specific resistances, to obtain full virulence for this bacterial type. When Vi phage II, by adaptation to Vi type E_7 , has obtained these two specific affinities, it must therefore attack also the Types E_1 and 29 (SCHOLTENS, 1955).

This hypothesis is supported by the fact that by transferring the specific resistance 29 with the natural phage 29' to strains of type E_1 cultures are obtained which, with the specific preparations of the system of CRAIGIE and FELIX, react like type E_7 .

A further example is as follows. Vi phage II F_2 attacks types F_1 and 29 in addition to its own type. Type F_1 can be converted into type F_2 with the latent phage f_1 or with the latent phage 29' which in serological and other respects agrees with phage f' . The specificity of this type is thus complex and built up from the resistance factors (F) and (29).

From old cultures of strains of type F_2 strains of type F_1 can often be isolated; type F_2 has a tendency to lose the specificity (29) and to degrade to type F_1 . Three cultures on Dorset medium made in 1951 from the type strain F_2 of FELIX appeared in 1954 to show microplaques with Vi phage E_7 at the critical test concentration. The strain appeared on examination to be degraded. The cultures were plated and sub-cultured from separate colonies. These sub-cultures appeared partly to belong to type F_2 , and partly to type F_1 . Moreover, some belonged to type 29. It is thus not only possible for the strains from type F_2 first to lose the specific resistance (29) and to go over to type F_1 , but perhaps they can also first lose the specific resistance F_1 and degrade into type 29.

The Joint Chairmen of the I.C.E.P.T. (International Committee of Enteric Phage Typing) have appointed a special subcommittee

to conduct co-ordinated investigations on an international basis into the Vi type groups C, D, E and F of CRAIGIE and FELIX. Here the relationship found at the National Institute for Public Health between the Types E₃, E₄ and the Type D₁, will be discussed. The complexity of the specific resistances of these types will be dealt with.

MATERIAL AND METHODS.

Technique. The technique described by CRAIGIE and FELIX (1947) was used throughout the experiments.

Bacterial Strains.

1. The strains E₄ (type strain), E₄ 491, E₄ 1779, E₄ 2576 and E₄ 2593 of DESRANLEAU.
2. The strains E₃ 1441, E₃ 1447 and E₃ 1814 of NICOLLE.
3. The type strains distributed by Dr FELIX.
4. The strains Ty 55-628, Ty 55-665 and Ty 55-711 of type D₁ and the strains Ty 55-737 and Ty 55-738 of type E₁ isolated at the National Institute of Public Health in Utrecht.

Phage preparations.

1. The typing phages distributed by Dr FELIX.
2. Preparation Vi phage II E₃ of DESRANLEAU.

THE COMPLEXITY OF THE PHAGE RESISTANCE OF VI TYPE E₃.

In 1944 a new Vi type *Salmonella typhi* was identified by DESRANLEAU (1947) in Canada. He was able to adapt the Vi phage II of CRAIGIE and YEN to the new type. The adaptation attacked not only the new type but also Type E₁. He therefore called the new type: Type E₃.

The following arguments may be put forward for the hypothesis that type E₃ possesses the specific resistances E₁ and D₁.

1. The adaptation of Vi phage II A to Type E₃ obtained by DESRANLEAU, attacks Type D₁ in addition to the types E₃ and E₁ at the critical test concentration.

2. By adapting Vi phage II D₁¹⁾, and also Vi phage II E₁ to

¹⁾ The preparation of Vi phage D₁ utilised in these experiments was obtained by regenerating the typing Vi phage II D₁ from a separate plaque on an a lysogenic strain of type D₁. The strains of types E₃ and E₄ were sensitive to the natural phage d₁ present in other preparations of Vi phage D₁.

the type E_3 of DESRANLEAU preparations were obtained that attack the Type E_3 of DESRANLEAU as well as the Vi types E_1 and D_1 .

If Vi phage II A is adapted to Type E_1 , a preparation is obtained after a single passage, which gives confluent lysis with Type E_1 at the critical test concentration; the adaptation to the Type D_1 needs more than one passage.

Parallel to this it was found that in adapting Vi phage II D_1 ¹⁾ to Type E_3 after a single passage an adaptation to Type E_3 was obtained; for the specific affinity that the Vi phage II has yet to attain, is the specific affinity E_1 ; which can be obtained after a single passage.

Starting from Vi phage E_1 in order to obtain an adaptation to Type E_3 we found that more passages were needed; the specific affinity D_1 to be acquired was obtained only after several passages.

3. Adaptation of Vi phage II D_1 to Type E_1 gives a phage preparation that is similar to an adaptation of Vi phage II to Type E_3 .

Vi phage II may lose or retain an affinity obtained by adaptation during a passage on another type which does not possess the corresponding specific resistance. The specific affinity for Type E_1 is lost under these circumstances, the specific affinity for Type D_1 is retained. Thus, in adapting Vi phage II D_1 to Type E_1 , a phage preparation is obtained, which has the specific affinities D_1 and E_1 . The fact that this adapted preparation attacks the Type E_3 at the critical test concentration argues for the complex structure (E_1 , D_1) of the specific resistance of Type E_3 .

4. Further arguments can be derived from the type transformation Type $E_1 \rightarrow$ Type E_3 with the natural phage d' from the Type D_1 .

FELIX and ANDERSON (1950) transformed Type E_1 with the natural phage d_1 into another Vi type which reacted with none of the preparations of the system of CRAIGIE and FELIX then known.

This type transformation was repeated. The artificial new type appeared to react with the preparation adapted to Type E_3 at the critical test concentration.

a) From mixed cultures of different strains of the Vi type E_1 with strains of the Vi type D_1 cultures of typhoid bacteria were obtained which in their behaviour with regard to the Vi phage II corresponded to the Vi type E_3 . However, in contrast with the

¹⁾ See note page 283.

naturally occurring Type E₃, they were lysogenic, and contained natural phage d'.

b) Strains of the Vi type E₁ were incubated in filtrates of 24 hr old broth cultures of strains of the Vi type D₁. After 2×24 hr incubation the cultures were plated, and subcultured from separate colonies. Some of the cultures obtained reacted with the preparations of the system of CRAIGIE and FELIX like type E₃. They were lysogenic and yielded the natural phage d'. In this they differed from the naturally occurring Type E₃.

Here the specific resistance (D₁) is transferred with the natural phage to bacteria of the Type E₁. The fact that these bacteria, which possess the specific resistances (D₁) and (E₁) behave with respect to the system of CRAIGIE and FELIX exactly like the type E₃, argues for the hypothesis that the specific resistance of Type E₃ is composed of the specific resistances E₁ and D₁.

THE RELATIONSHIP BETWEEN THE VI TYPES E₄, E₃, E₁ AND D₁.

Type E₄ was found by DESRANLEAU (1947) and diagnosed with an adaptation of his phage Q-1467-43, a phage related serologically to Vi phage II. He found that this preparation lysed also Types E₁, E₂ and E₃.

The strains of Type E₄ distributed by DESRANLEAU to the members of the subcommittee, were sensitive to all the preparations of Vi phage II adapted to types of the E group; with the critical test concentration of these preparations microplaques developed which were only visible with the plate microscope (table 1).

The strains of the Vi type E₃ received from NICOLLE were sensitive to Vi phages II, E₃ and E₄ but wholly resistant to the critical test concentration of further adapted preparations.

EDWARDS and WILSON in U.S.A. and SCHOLTENS in Holland ¹⁾ succeeded simultaneously (summer 1955) in obtaining adaptations of Vi phage II A to Type E₄. In contrast with the preparation of DESRANLEAU ²⁾ derived from phage (Q-1467-43) the preparations derived from Vi phage II did not attack type E₂.

It appeared that not only the preparations distributed to the members of the subcommittee by SCHOLTENS but also those distributed by EDWARDS and WILSON, possessed an increased virulence for the types E₁ and D₁. In a concentration of $10 \times$ the critical

¹⁾ In Holland adaptations to all the strains of DESRANLEAU were obtained.

²⁾ Cited from DESRANLEAU (1947).

TABLE 1.
Reactions of Vi-type group E of *Salmonella typhi*.

Vi-type strains	Adapted preparations of Vi phage II							
	E ₁	E ₂	E ₃	E ₄	E ₅	E ₆	E ₇	E ₈
A	cl	cl	cl	cl	cl	cl	cl	cl
D ₁	—	—	cl	cl	—	—	—	—
E ₁	cl	cl	cl	cl	cl	cl	cl	cl
E ₂	—	cl	—	—	—	—	—	—
E ₃	—	—	cl	cl	—	—	—	—
E ₄	+2 $\mu\mu$	+2 $\mu\mu$	+4 $\mu\mu$ ¹⁾	cl	+2 $\mu\mu$	+1 $\mu\mu$	$\pm\mu\mu$	$\pm\mu\mu$
E ₅	—	—	—	—	cl	—	—	—
E ₆	—	—	—	—	—	cl	—	—
E ₇	—	—	—	—	—	—	cl	—
E ₈	—	—	—	—	—	—	—	cl

cl = confluent lysis. $\mu\mu$ = plaques as dustlike particles to be observed with the colony microscope.

+4 = a number of very small plaques, that would be confluent, if they were larger.

test concentration the preparations of EDWARDS and WILSON gave confluent lysis with the strains of Type D₁. With the critical test concentration reactions of +3 and +2 were noted. The preparations obtained by SCHOLTENS showed confluent lysis in the critical test concentration with strains of type D₁. In these preparations the virulence corresponding to this specific resistance was fully developed (table 2).

In view of the above it may be accepted that Type E₄ possesses the specific resistance (D₁) in addition to the specific resistance (E). The fact that the Type E₄ possesses these two specific resistances does not entirely account, however, for its behaviour with respect to Vi phage II. For Type E₄ is not completely sensitive to phage preparations that have the specific virulences E₁ and D₁ alone.

This was clearly shown with the preparations obtained by adapting Vi phage II D₁ to Type E₁, but less clearly with the preparation DESRANLEAU obtained in adapting Vi phage II to Type E₃.

We did not succeed, starting from the preparations Vi phage II D₁ in obtaining an adapted preparation to Type E₄ by one or two passages on strains of Type E₄, nor when starting from Vi phage II D₂, D₄ or D₅.

¹⁾ The reaction may be more conspicuous, and difficult to discern from the reaction upon type E₃.

TABLE 2.

Reactions of strains of Vi-types A, D₁, E₁, E₃ and E₄ to different adapted preparations of Vi phage II (Readings were made after 24 hrs incubation).

Strains	Adapted preparations of Vi phage II obtained by:								
	SCHOLTENS			EDWARDS			DES-RAN- LEAU		
	E ₄	E ₄ , 2593		E ₄ , 9	E ₄ , 20		E ₃	A	E ₁ D ₁
	10 × ctc ctc	10 × ctc ctc	10 × ctc ctc	10 × ctc ctc	10 × ctc ctc	10 × ctc ctc	10 × ctc ctc	100 × ctc ctc ctc	
A (type strain)	cl cl	cl cl	cl cl	cl cl	cl cl	cl cl	cl cl	cl cl	cl
E ₁ (type strain)	cl cl	cl cl	cl cl	cl cl	cl cl	cl cl	cl cl	± cl	—
D ₁ (55-628)	cl cl	cl cl	scl +3	scl +3	scl +3	scl +3	scl scl	± —	cl
E ₃ (1441, NI-COLLE)	cl cl	cl scl	cl +3	cl +3	cl scl	— — —			
E ₄ (491, DES-RANLEAU)	cl cl	cl scl	cl scl	cl scl	cl +3μ	— — —			

ctc = critical test concentration; cl = confluent lysis; cl⁻ = confluent lysis, whereby the plaques can be distinguished, especially in the margin. scl = semiconfluent lysis.

+1, +2, +3 = an increasing number of plaques; +4 = a number of small plaques, that would be confluent, if they were larger.

μ = plaques as dustlike particles to be observed with the colony microscope.

The critical test concentrations were taken as indicated by the authors who obtained the preparations.

preparations:	critical test concentrations:	obtained with strains:
E ₄ ¹⁾	10 ⁻⁴	E ₄ (type strain)
E ₄ 2593	1/3 × 10 ⁻⁴	E ₄ (2593)
E ₄ 9	10 ⁻⁴	
E ₄ 20	10 ⁻³	
E ₃	10 ⁻³	

Summary and Conclusions.

- Vi phage II E₃ gives in the critical test concentration cross reactions with the Types E₁ and D₁; the specific resistance of Type E₃ is complex, and composed of the specific resistances E₁ and D₁.
- Vi phage II E₄ gives in the critical test concentration cross reactions with the types E₃, E₁ and D₁.

¹⁾ This preparation E₄ was obtained by further passages on type strain E₄ from a preparation E_{4a} distributed to the members of the Subcommittee. In this preparation E_{4a} the affinity for type D₁ was very incompletely developed.

3. Type E_4 differs from Type E_3 in phage reactions in that it is more sensitive to preparations of Vi phage II with the specific affinity E_1 and more resistant to preparations of Vi phage II with the specific affinities (E_1) and (D_1).
4. It is more difficult to adapt Vi phage II to Type E_4 than to Type E_3 , not only if one starts from Vi phage II A (DESRANLEAU, 1947) but also if one starts from Vi phage II D_1 .

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ÜBER HAEMAGGLUTININE IN PFLANZENEXTRACTEN

von

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Schon seit langem ist bekannt, dass Pflanzenstoffe Erythrocyten zu agglutinieren vermögen. Die agglutinierende Fähigkeit von Ricin wurde durch STILLMARK (1887) im Laboratorium von KOBERT entdeckt. Man glaubte, darin eine echte Toxinwirkung in vitro sehen zu dürfen. Schon damals zeigte es sich, dass die Erythrocyten verschiedener Tierarten und innerhalb der gleichen Species verschiedener Individuen nicht gleichmässig empfindlich waren. Sehr wichtig war auch die Beobachtung, dass normale Sera eine gewisse agglutinationshemmende Wirkung auszuüben vermögen. Die haemagglutinierende Fähigkeit des Ricins geht durch Erwärmen auf Coagulationstemperatur verloren. Die Haemagglutination durch Abrin wurde durch KOBERT (1889) und HELLIN (1891) beschrieben. Auch Crotin wirkt in schwachen Concentrationen haemagglutinierend, in stärkeren haemolytisch. LANDSTEINER und STANKOVIC (1906) zeigten später, dass Ricin und Abrin durch Proteine gebunden wird. Aus *Amanita muscaria* und *Amanita solitaria* wurden durch FORD (1907, 1908) und ABEL und FORD (1908) wirksame Haemagglutinine isoliert, und aus der Euphorbiaceae *Hura crepitans* erhielt RICHET (1909) ein Toxin und Haemagglutinin, das er Crepitin nannte.

Die ersten Untersuchungen über Pflanzenhaemagglutinine, wobei es sich nicht um Toxine handelte, stammen von LANDSTEINER und RAUBITSCHKE (1908), VON EISLER und VON PORTHEIM (1908), KOBERT (1909), WIENHAUS (1909) und ASSMANN (1911). Das Phasin (Haemagglutinine aus verschiedenen Bohnenarten) wird nach LANDSTEINER und RAUBITSCHKE (1908) durch Kochen zerstört,

WIENHAUS (1909) fand schon 90° ausreichend hierfür. Den hemmenden Einfluss von Pepton und Serumeiweiss auf die Haemagglutination durch Pflanzenstoffe untersuchte ausser LANDSTEINER auch noch RAUBITSCHKE (1909).

STILLMARK (1887), LIEBERMANN (1907) und GUYOT (1909) wiesen nach, dass Phytotoxine mit der Membran der Erythrocyten eine Bindung eingehen.

In jüngerer Zeit wurde die Haemagglutination durch Pflanzenextracte durch BOYD (1947), BIRD (1952) u.a. wieder aufgenommen, wobei sich das interessante Ergebnis herausstellte, dass in einzelnen Fällen hierdurch eine Bestimmung menschlicher Blutgruppen möglich wurde. Erwähnt sei ferner, dass in Versuchen von COLLIER und TIGGELMAN-VAN KRUGTEN einige stark agglutinierende Pflanzenextracte durch das Keratinhydrolysat Detoxin in ihrer haemagglutinierenden Fähigkeit gehemmt wurden.

In fast allen bisherigen Untersuchungen wurden ausschliesslich Haemagglutinine aus Samen untersucht. Im Nachfolgenden sollte vor Allem festgestellt werden, ob auch Blätter verschiedener Pflanzenarten imstande waren, verschiedene Blutarten zu haemagglutinieren. Nur gelegentlich wurden auch Extracte von Blumen und Samen geprüft.

ALLGEMEINE TECHNIK.

Die Pflanzen wurden im Waringblender mit der 20-fachen Menge steriler physiologischer Kochsalzlösung zerkleinert. Die Flüssigkeit wurde durch Papier filtriert und im Eisschrank aufbewahrt, wo sie meistens wochenlang unverändert blieb. Nur in einzelnen Fällen nahm die Wirkung ab. Falls nötig, wurden die Extracte neutralisiert.

Zur Untersuchung kam Blut von Menschen (O, A, B), Hammel, Meerschweinchen und Huhn. Die Erythrocyten wurden dreimal in physiologischer Kochsalzlösung gewaschen und im Eisschrank bewahrt. Kurz vor Gebrauch wurde eine 1%ige Suspension in Kochsalzlösung hergestellt. Das Alter des Blutes (1–4 Tage) schien keinen Einfluss auszuüben.

Die Agglutinationsversuche wurden in sterilen Wassermann-Röhrchen aus Pyrexglas durchgeführt.

Wenn in einzelnen Fällen der Eindruck hervorgerufen wurde, dass menschliches Blut verschiedener Gruppen sehr deutlich verschieden stark beeinflusst würde, so wurden die Versuche mit einer

Serie von Blutmustern der verschiedenen Blutgruppen erneut untersucht.

TECHNIK DES HAEMAGGLUTINATIONSVERSUCHES.

In einer Reihe von Röhrchen wurden fallende Verdünnungen des Pflanzenextractes in Zweierpotenzen in einem Volumen von 0,5 ml hergestellt. Hierzu wurde je 0,5 ml der verschiedenen Blutsuspensionen gebracht. Nach Durchschütteln blieben die Röhrchen bei Zimmertemperatur (22–25°C.) stehen. Abgelesen wurde nach völliger Sedimentierung der Erythrocyten nach etwa 1½–2 Stunden. Das Ergebnis wurde als stark positiv (+++) bezeichnet, wenn die Erythrocyten die Kuppe des Röhrchens als dicker Film bedeckten, als negativ (0), wenn auf dem Boden ein roter Knopf mit scharfem Rande zu sehen war.

Die Berechnung der Verdünnungen erfolgte stets für die Endconcentration im Hinblick auf das Gesamtvolumen. Waren also im ersten Röhrchen 0,5 ml 5%iger Pflanzenextract und 0,5 ml Blutsuspension, so wurde als Titer des Extractes 1/40 angegeben.

ERGEBNISSE DER VERSUCHE.

Die Resultate sind in der Tabelle 1 zusammengefasst. Es geht daraus hervor, dass von den untersuchten 313 Extracten 87 (= 27,8%) vollkommen unwirksam waren und in der Concentration von 1/40 keine der untersuchten Blutarten beeinflussten. Dies war beispielsweise der Fall bei 1 von 17 *Rubiaceae* (= 6%), 14 von 83 *Leguminosae* (= 17%), 5 von 22 *Malvaceae* (= 23%), 11 von 42 *Euphorbiaceae* (= 26%), 5 von 18 *Verbenaceae* (= 28%), 5 von 12 *Apocynaceae* (= 42%), 3 von 6 *Palmae* (= 50%), 8 von 15 *Acanthaceae* (= 53%) und 23 von 30 *Gramineae* (= 77%).

Weiterhin verursachten von den 313 Extracten 114 (= 36,4%) bei allen 6 untersuchten Blutarten Haemagglutination. Dies zeigte sich beispielsweise bei 15 von 17 *Rubiaceae* (= 88%), 5 von 6 *Combrretaceae* (= 83%), 24 von 42 *Euphorbiaceae* (= 57%), 40 von 83 *Leguminosae* (= 48%), 8 von 22 *Malvaceae* (= 36%), 2 von 6 *Palmae* (= 33%), 2 von 15 *Acanthaceae* (= 13%), 1 von 18 *Verbenaceae* (= 6%), 1 von 30 *Gramineae* (= 3%) und 0 von 12 *Apocynaceae* (0%).

Von den 313 Extracten haemolysierten 22 (= 7,0%) alle 6 untersuchten Blutarten. Dies war u.a. der Fall bei 6 von 18 *Verbenaceae* (= 33%), 2 von 22 *Malvaceae* (= 9%), 5 von 83 *Leguminosae*

TABELLE 1.

Haemagglutination der Erythrocyten von Huhn, Meerschweinchen, Ham-
mel, Mensch O, A und B durch 313 Pflanzenextrakte.

Legende: Die Ziffern stellen die reziproken Werte der Endconcentrationen dar, wodurch komplette Haemagglutination hervorgerufen wird. Die eingeklammerten Ziffern (...) bedeuten eine nur partielle, aber doch deutliche Haemagglutination bezw. Haemolyse.

Fettdruck bedeutet Lyse.

Haemagglutination der Erythrocyten von							
Untersuchte Species:	Material:	Huhn	Meer- schw.	Ham- mel	Mensch O	Mensch A	Mensch B
Acanthaceae:							
1. <i>Asystasia gangetica</i>	Blatt	80	(160)	80	160	80	(80)
2. " "	Blume weiss	0	0	0	0	0	0
3. " "	Blume lila	0	0	0	0	0	0
4. <i>Barleria cristata</i>	Blatt	0	0	0	0	0	0
5. <i>Beloperona guttata</i>	Blatt	320	640	0	160	160	160
6. " "	Blume	(2560)	5120	0	2560	1280	1280
7. <i>Blechnum brownii</i>	Blatt	0	0	0	0	0	0
8. <i>Eranthemum tricolor</i>	Blatt	0	0	0	0	0	0
9. <i>Justicia pectoralis</i>	Blatt	0	0	0	0	0	0
10. <i>Megaskepasma erythrochlamys</i>	Blatt	80	80	80	80	80	80
11. " "	Blume rot	0	160	0	40	(160)	80
12. <i>Ruellia tuberosa</i>	Blatt	0	0	0	0	0	0
13. <i>Thunbergia alata</i>	Blatt	0	0	0	0	0	0
14. <i>Trichanthera gigantea</i>	Blatt	10240	0	0	0	0	0
15. " "	Blume	40	0	0	0	0	0
Amaranthaceae:							
16. <i>Alternanthera spec.</i>	Blatt	0	0	0	0	0	0
Anacardiaceae:							
17. <i>Mangifera indica</i>	Blatt	80	160	80	80	0	160
Anonaceae:							
18. <i>Anona muricata</i>	Blatt	320	(640)	640	160	640	80
Apocynaceae:							
19. <i>Allamanda cathartica</i>	Blatt	0	0	0	(40)	(40)	(40)
20. " "	Blume	80	160	160	160	320	160
21. " "	Samen	0	0	0	0	0	0
22. " "	Frucht- fleisch	0	0	0	0	0	0
23. <i>Cryptostegia madagascariensis</i>	Blatt	0	0	(40)	0	0	0
24. " "	Frucht	40	320	80	0	40	40
25. <i>Lochnera rosea</i>	Blatt	0	160	0	40	40	0
26. " "	Blume rot	0	160	40	80	80	80
27. <i>Plumeria alba</i>	Blatt	0	0	0	0	0	0
28. <i>Strophantus spec.</i>	Blatt	0	0	0	0	0	0
29. <i>Tabernaemontana coronaria</i>	Knospe	0	0	0	0	(40)	(40)
30. " "	Blatt	0	0	0	0	0	0
Araceae:							
31. <i>Amorphophallus variabilis</i>	Blatt	0	0	40	0	40	0
32. <i>Dieffenbachia picta</i>	Blatt	0	80	40	40	40	0
33. <i>Montrichardia arborescens</i>	Blatt	0	80	0	0	0	0
Araliaceae:							
34. <i>Aralia guilfoylei</i>	Blatt	1280	2560	1280	1280	2560	2560
Bombacaceae:							
35. <i>Ceiba pentandra</i>	Blatt	0	40	(40)	0	0	0
Bromeliaceae:							
36. <i>Ananas comosus</i>	Blatt	0	0	0	0	0	0
Cactaceae:							
37. <i>Cactus caesijs</i>	Stamm	0	0	0	0	0	0

Untersuchte Species:	Material:	Haemagglutination der Erythrocyten von					
		Huhn	Meerschw.	Ham-mel	Mensch O	Mensch A	Mensch B
38. <i>Cereus hexagonus</i>	Stamm	0	(40)	0	0	0	0
39. <i>Hylocereus lemairei</i>	Stamm	0	0	0	0	0	0
40. <i>Nopalea coccinellifera</i>	Stamm	0	0	0	(80)	40	0
41. <i>Rhipsalis cassytha</i>	Stamm	320	320	160	160	160	160
42. „	Samen	80	40	40	40	40	40
Caprifoliaceae:							
43. <i>Lonicera japonica</i>	Blatt	320	320	160	320	80	320
44. <i>Sambucus canadensis</i>	Blatt	0	80	0	80	80	80
Caricaceae:							
45. <i>Carica papaja</i>	Blatt	0	80	80	160	320	160
Caryophyllaceae:							
46. <i>Drymaria cordata</i>	Blatt	0	640	0	0	0	0
Combretaceae:							
47. <i>Combretum rotundifolium</i>	Blatt	2560	10240	5120	640	320	320
48. <i>Laguncularia racemosa</i>	Blatt	1280	320	1280	1280	320	320
49. „	Blume	1280	1280	1280	80— (640)	(40)— —160	40— —320
50. <i>Quisqualis indica</i>	Blatt	1280	10240	320	320	160	320
51. <i>Terminalia catappa</i>	Blatt	(640)	320	160	160	160	160
52. <i>Terminalia dichotoma</i>	Blatt	1280	5120	(2560)	(1280)	160	(1280)
Commelinaceae:							
53. <i>Commelina nudiflora</i>	Blatt	320	0	0	0	0	0
Compositae:							
54. <i>Eupatorium triplinerve</i>	Blatt	0	160	0	0	0	0
55. <i>Vernonia cinerea</i>	Blatt	0	0	0	0	0	0
Euphorbiaceae:							
56. <i>Acalypha bajourii</i>	Blatt	1280		1280	1280	1280	640
57. <i>Acalypha hispida</i>	Blatt	5120	5120	2560	2560	1280	1280
58. „	Blume	2560		1280	2560	1280	1280
59. <i>Acalypha macrostachya</i>	Blatt	5120	1280	1280	2560	1280	1280
60. „	Blume	2560	320	320	(640)	320	320
61. <i>Acalypha spec.</i>	Blatt	5120	2560	1280	1280	2560	2560
62. „	Blume						
	+Frucht	2560	1280	640	640	1280	1280
63. <i>Aleurites moluccana</i>	Blatt	20480		20480	5120	10240	20480
64. „	Blume	20480		10240	5120	10240	(20480)
65. <i>Aleurites montana</i>	Blatt	20480	10240	1280	(10240)	5120	2560
66. „	Frucht-fleisch	10240	2560	320	2560	2560	640
67. <i>Caperonia palustris</i>	Blatt	10240	2560	1280	1280	2560	5120
68. „	Blume						
	+Frucht	5120	(1280)	1280	1280	(5120)	2560
69. <i>Croton hirtus</i>	Blatt	0		0	0	0	0
70. „	Blume						
	+Frucht	0	0	0	0	0	0
71. „	Samen	0	0	0	0	0	0
72. <i>Croton lobatus</i>	Blatt	0	0	0	0	0	0
73. „	Samen	0	0	0	0	0	0
74. <i>Euphorbia geniculata</i>	Blatt	640	640	80	80	160	80
75. „	Blume	0	(160)	0	0	0	0
	+Samen						
76. <i>Euphorbia heterophylla</i>	Blatt	0	0	0	0	0	0
77. „	Samen	0	0	0	0	0	0
78. „	Blume	0	0	0	0	0	0
79. <i>Euphorbia hirta</i>	Blatt	2560	2560	320	640	640	(640)
80. „	Samen	640	320	160	(320)	(320)	320
81. <i>Euphorbia hypericifolia</i>	Blatt	1280	2560	640	640	(1280)	640
82. „	Samen	640	320	160	160	160	160
83. <i>Euphorbia prostrata</i>	Blatt						
	+Samen	1280	1280	160	320	320	320
84. <i>Hura crepitans</i>	Blatt	0	0	0	0	0	0
85. <i>Jatropha gossypifolia</i>	Blatt	(10240)	1280	(640)	1280	1280	1280
86. „	Samen	(640)	320	320	320	160	160
87. <i>Jatropha hastata</i>	Blatt	0	0	0	0	0	0
88. <i>Jatropha podagrica</i>	Blatt	0	0	(160)	0	0	0
89. „	Frucht	0	0	0	0	0	0
90. <i>Manihot utilissima</i>	Blatt	2560	5120	(320)	640	320	320

Untersuchte Species:	Material:	Haemagglutination der Erythrocyten von					
		Huhn	Meersch.	Ham-mel	Mensch O	Mensch A	Mensch B
91. <i>Pedilanthus tithymaloides</i>	Blatt	320	640	0	640	320	160
92. "	Samen	0	160	0	0	0	0
93. <i>Phyllanthus disciculatus</i>	Blatt	640	640	(320)	0	160	(320)
94. <i>Phyllanthus niruri</i>	Blatt+						
	Samen	1280	(640)	0	160	320	(640)
95. <i>Phyllanthus urinaria</i>	Blatt	2560	1280	(1280)	1280	640	640
96. <i>Ricinus communis</i>	Blatt	640	0	(160)	0	0	0
97. <i>Sebastiania corniculata</i>	Blatt	1280	1280	320	320	640	320
Gramineae:							
98. <i>Acroceras macrum</i>	Blatt	0	0	0	0	0	0
99. <i>Andropogon citratus</i>	Blatt	40	40	0	0	0	0
100. <i>Andropogon nardus</i>	Blatt	(40)	0	0	0	0	0
101. <i>Andropogon sorghum</i>	Blatt	0	0	0	0	0	0
102. <i>Axonopus affinis</i>	Blatt	0	0	0	0	0	0
103. <i>Axonopus compressus</i>	Blatt	0	0	0	0	0	0
104. <i>Bambusa multiplex</i>	Blatt	0	0	0	0	0	0
105. <i>Bambusa vulgaris</i>	Blatt	0	0	0	0	0	0
106. <i>Brachiaria purpurescens</i>	Blatt	0	0	0	0	0	0
107. <i>Chloris guayana</i>	Blatt	0	0	0	0	0	0
108. <i>Cynodon dactylon</i>	Blatt	0	0	0	0	0	0
109. <i>Dendrocalamus asper</i>	Blatt	0	0	0	0	0	0
110. <i>Digitaria decumbens</i>	Blatt	0	0	0	0	0	0
111. <i>Digitaria horizontalis</i>	Blatt	0	0	0	0	0	0
112. <i>Eleusine indica</i>	Blatt	0	0	0	0	0	0
113. <i>Gigantochloa apus</i>	Blatt	0	0	0	0	0	0
114. <i>Gynerium sagittatum</i>	Blatt	0	0	0	0	0	0
115. <i>Ischaemum aristatum</i>	Blatt	0	0	0	0	0	0
116. <i>Ischaemum timorense</i>	Blatt	0	0	0	0	0	0
117. <i>Melinis minutiflora</i>	Blatt	0	40	0	0	0	0
118. <i>Oryza sativa</i>	Blatt	0	0	0	0	0	0
119. <i>Panicum coloratum</i>	Blatt	160	160	160	160	160	160
120. <i>Paspalum dilatatum</i>	Blatt	0	0	0	0	0	0
121. <i>Paspalum notatum</i>	Blatt	0	0	0	0	0	0
122. <i>Pennisetum purpureum</i>	Blatt	0	0	0	0	0	0
123. <i>Saccharum officinarum</i>	Blatt	0	0	0	0	0	0
124. <i>Stenolaphrum secundatum</i>	Blatt	0	(80)	0	0	0	0
125. <i>Tripsacum laxum</i>	Blatt	40	(80)	80	0	80	40
126. <i>Veliveria zizanioides</i>	Blatt	0	0	0	0	0	0
127. <i>Zea mais</i>	Blatt	160	320	320	320	320	320
Labiatae:							
128. <i>Orthosiphon grandiflorus</i>	Blatt	0	0	0	0	0	0
129. "	Blume	0	0	0	0	0	0
Leguminosae:							
130. <i>Adenanthera pavonina</i>	Blatt	0	0	0	(80)	(80)	(80)
131. <i>Aeschynomene sensitiva</i>	Blatt	(80)	40	40	80	160	(80)
132. "	Samen	(40)	(40)	0	0	0	0
133. <i>Andira inermis</i>	Blatt	80	1280	80	80	80	80
134. <i>Arachis hypogaea</i>	Blatt	0	0	0	0	0	0
135. "	Frucht	0	0	0	0	0	0
136. <i>Bauhinia variegata</i>	Blatt	160	320	320	320	320	320
137. <i>Brounea hybrida</i>	Blatt	20480	40960		10240		2560
138. <i>Caesalpinia coriacea</i>	Blatt	20480	10240	(640)	640	320	320
139. <i>Caesalpinia pulcherrima</i>	Blatt	1280	(1280)	1280	320	320	320
140. <i>Calliandra brevipes</i>	Blatt	2560	640	2560	2560	320	(1280)
141. <i>Calopogonium mucunoides</i>	Blatt	0	0	0	0	0	0
142. "	Schote	0	0-80	0	0-40	0-80	0-80
143. <i>Camoënsia maxima</i>	Blatt	(160)	160	160	160	160	(160)
144. <i>Canavalia ensiformis</i>	Blatt	0	40	0	0	0	0
145. "	Samen	20480	20480-	20480	5120	2560	5120
			-163840				
146. <i>Cassia alata</i>	Blatt	40	0	0	(160)	(80)	160
147. "	Samen	40	40	80	80	80	80
148. <i>Cassia biflora</i>	Blatt	2560	5120	320	640		(640)
149. <i>Cassia fistulata</i>	Blatt	0	0	0	0	80	40
150. <i>Cassia fruticosa</i>	Blatt	0		80	0	0	0
151. <i>Cassia occidentalis</i>	Blatt	1280	80	0	0	0	0
152. <i>Cassia reticulata</i>	Blatt	0-160	40	0	0-40	0	0
153. <i>Cassia spectabilis</i> I	Blatt	160	320	320	320	320	320
154. " II	Blatt	0	0	40	0	0	0
155. <i>Cassia tora</i>	Blatt	0	0	0	0	0	0
156. <i>Centrosema virginianum</i>	Blatt	0	(80)	0	0	0	0

Untersuchte Species:	Material:	Haemagglutination der Erythrocyten von					
		Huhn	Meerschw.	Hammel	Mensch O	Mensch A	Mensch B
157. <i>Clitoria ternatea</i>	Blatt	0	0	40	40	0	40
158. "	Blume	40	80	80	80	80	80
159. "	Schote	0	0	(80)	80	160	160
160. <i>Crotalaria anagyroides</i>	Blatt	640	2560	1280	1280	2560	2560
161. <i>Crotalaria retusa</i>	Blatt	40	80	160	640	1280	640
162. "	Blume	40	80	40	40	40	40
163. "	Schote	0	0	0	0	0	0
164. <i>Crotalaria striata</i>	Blatt	(80)	80	80	80	(160)	(160)
165. "	Blume	(40)	(40)	(40)	0-40	0-640	0-(80)
166. "	junge Samen	0	(40)	(40)	40	320	(40)
167. "	alte Samen	(80)	160	160	160	(320)	160
168. <i>Cynometra cauliflora</i>	Blatt	10240	20480	20480	5120	5120	20480
169. <i>Delonix regia</i>	Blatt	2560	2560	2560	5120	5120	2560
170. <i>Derris elliptica</i> (1)	Blatt	2560	20480	10240	640	640	640
(2)	Blatt	5120	2580	320	640	320	160
171. <i>Desmanthus virgatus</i>	Blatt	0	0	0	0	0	0
172. <i>Desmodium asperum</i>	Blatt	0	0	0	0	0	0
173. <i>Desmodium frutescens</i>	Blatt	80	320	80	80	0	0
174. "	Samen	0	80	(80)	80	0	(80)
175. <i>Desmodium procumbens</i>	Blatt	40	40	40	40	40	0
176. <i>Desmodium triflorum</i>	Blatt	320	320	(640)	640	640	640
177. <i>Enterolobium cyclocarpum</i> (1)	Blatt	20480	20480	20480	20480	20480	20480
(2)	Blatt	10240	163920	5120	81920	81920	163920
178. <i>Erythrina glauca</i>	Blatt	0	0	0	0-40	0	0
179. <i>Erythrina poeppigiana</i>	Blatt	80	0	0	0	0	0
180. <i>Gliricidia septium</i>	Blatt	0	0	0	0	0	0
181. <i>Glycine maxima</i>	Blatt	0	0	0	(40)	0	0
182. <i>Indigofera subulata</i>	Blatt	1280	160	1280	320	160	320
183. "	Samen	(2560)	(2560)	(2560)	(2560)-	160-	320-
					-10240	-2560	-5120
184. <i>Inga spec.</i> I (1)	Blatt	10240	20480	10240	20480	2560	
(2)	Blatt	5120	5120	5120	(5120)	2560	1280
185. <i>Inga spec.</i> II	Blatt	20480	81920	5120	10240	163920	81920
186. <i>Inga spec.</i> III	Blatt	640	20480	10240	320	640	320
187. <i>Inocarpus edulis</i>	Blatt	0	0	0	0	0	0
188. <i>Leucaena glauca</i>	Blatt	160	80	80	80	80	(160)
189. <i>Mimosa invisa</i>	Blatt	(320)	320	80	1280	(160)	(320)
190. <i>Mimosa pudica</i>	Blatt	160	160	80	80	160	80
191. <i>Mucuna sioanei</i>	Blatt	1280	640	1280	10240	5120	(5120)
192. "	Samen	80	320	640	320	320	320
193. <i>Myroxylon pereirae</i>	Blatt	0	0	0	0	0	0
194. <i>Peltophorum ferrugineum</i> (1)	Blatt	10240	2560	1280	1280	1280	640
(2)	Blatt	20480	20480	20480	10240	20480	10240
195. <i>Phaseolus lunatus</i>	Samen	0	80	0	0-80	10240	0-160
196. <i>Pithecolobium dulce</i>	Blatt	1280	2560	1280	640	640	1280
197. <i>Pithecolobium saman</i>	Blatt	1280	1280	320	1280	640	1280
198. <i>Pueraria phaseoloides</i>	Blatt	0	0	0	0	0	0
199. <i>Rhynchosia minima</i>	Blatt	160	80	320	160	40	80
200. <i>Saracca indica</i> (1)	Blatt	5120	10240	640	2560	1280	640
(2)	Blatt	10240	20480	10240	2560	1280	1280
201. "	Samen	1280	2560	2560	1280	320	320
202. <i>Sesbania grandiflora</i>	Blatt	320	20480	320	320	640	
203. "	Blume	80	80	40	80	80	
204. <i>Sesbania sericea</i>	Blatt	80	160	80	80	160	160
205. "	Schote	640	2560	640	640	1280	2560
206. <i>Tamarindus indica</i>	Blatt	640	640	640	(2560)	640	640
207. <i>Tephrosia candida</i>	Blatt	80	160	(160)	2560	2560	2560
208. "	Blume	80	80	160	160	80	80
209. "	Schote	0	160	160	320	160	160
210. <i>Tephrosia zollingeri</i>	Blatt	160	2560	2560	2560-	80-	80-
					-5120	-5120	-5120
211. <i>Vigna sinensis</i>	Blatt	0	40	0	0	0	0
212. "	Samen	0	0	0	0	0	0
Lythraceae:							
213. <i>Lagerstroemia indica</i>	Blatt	80	80	(160)	80	80	0
Malpighiaceae:							
214. <i>Galphimia glauca</i>	Blatt	(2560)	320	1280	640	320	320
215. "	Blume	640	640	5120	640	640	320
216. <i>Malpighia puniceifolia</i>	Blatt	0	0	0	0	40	(40)

Untersuchte Species:	Material:	Haemagglutination der Erythrocyten von					
		Huhn	Meersch. schw.	Ham- mel	Mensch O	Mensch A	Mensch B
Malvaceae:							
217. <i>Gossypium barbadense</i>	Blatt	80	(160)	(320)	80	80	(80)
218. <i>Hibiscus abelmoschus</i>	Blatt	80	80	40	40	40	40
219. <i>Hibiscus esculentus</i>	Blatt	0	40	0	(80)	(40)	0
220. "	Frucht	0	40	40	0	0	0
221. <i>Hibiscus rosa sinensis</i>	Blatt	0	80	40	40	40	40
222. "	Blume rot	0	0	0	0	0	0
223. "	Blume rosa	0	0	0	0	0	0
224. "	Blume gelb- orange	0	320	40	0	0	0
225. <i>Hibiscus schizopetalus</i>	Blatt	0	0	0	0	0	0
226. <i>Hibiscus sabdariffa</i>	Blatt	0	80	40	40	40	40
227. "	Frucht	160	320	160	80	80	80
228. <i>Hibiscus sororius</i>	Blatt	0	0	0	0	0	0
229. <i>Malachra alceifolia</i>	Blatt	(160)	80	40	(320)	1280	(160)
230. <i>Malachra radiata</i>	Blatt	(160)	80	80	1280	5120	2560
231. <i>Malva viscosa</i>	Blatt	0	0	0	0	0	0
232. <i>Sida acuta</i>	Blatt	640	320	160	2560	20480	2560
233. <i>Sida glomerata</i>	Blatt	320	320	320	320	640	(640)
234. "	Samen	(80)	80	40	(40)	(80)	(80)
235. <i>Sida rhombifolia</i>	Blatt	(640)	80	160	(1280)	1280	640
236. <i>Urena lobata</i> I	Blatt	40	640	320	160	40	0
237. <i>Urena lobata</i> II	Blatt	10240	(20480)	20480	(20480)	(20480)	2560
238. "	Samen	2560	1280	2560	320	5120	320
Musaceae:							
239. <i>Musa paradisiaca</i>	Blatt	0	40	0	40	160	0
Myrtaceae:							
240. <i>Eucalyptus spec.</i>	Blatt	10240	2560	2560	5120	2560	2560
Nyctaginaceae:							
241. <i>Bougainvillea glabra</i>	Blatt	40	160	160	80	80	40
242. <i>Mirabilis jalapa</i>	Blatt	40	0	80	(80)	80	40
Palmae:							
243. <i>Arenga pumata</i>	Blatt	1280	2560	2560	(5120)	2560	640
244. <i>Caryota mitis</i>	Blatt	0	0	0	0	0	(40)
245. <i>Cocos nucifera</i>	Blatt	2560	5120	2560	5120	5120	640
246. <i>Elaeis guineense</i>	Blatt	0	0	0	0	0	0
247. <i>Ptychospermum macarthurii</i>	Blatt	0	0	0	0	0	0
248. <i>Raphis flabelliformis</i>	Blatt	(320)	320	320	320	320	(160)
Papaveraceae:							
249. <i>Agremone mexicana</i>	Blatt	0	80	0	0	0	0
250. "	Frucht	640	(640)	160	80	80	320
Phytolaccaceae:							
251. <i>Microtea debilis</i>	Blatt	40	160	(160)	80	(160)	80
252. <i>Peteveria alliacea</i>	Blatt	0	0	0	0	0	0
Piperaceae:							
253. <i>Piper spec.</i>	Blatt	0	0	0	0	0	0
Plumbaginaceae:							
254. <i>Plumbago capensis</i>	Blatt	80	320	(640)	160	80	(320)
Polygalaceae:							
255. <i>Securidaca paniculata</i>	Blatt	0	0	0	0	0	0
Polygonaceae:							
256. <i>Antigonon leptopus</i>	Blatt	320	2560	80	320	1280	160
257. <i>Coccoloba uvifera</i>	Blatt	1280	1280	2560	2560	5120	2560
Portulacaceae:							
258. <i>Portulaca grandiflora</i>	Blatt	0	40	0	0	0	0
Rhamnaceae:							
259. <i>Ziziphus jujuba</i>	Blatt	1280	5120	2560	320	160	320

Haemagglutination der Erythrocyten von							
Untersuchte Species:	Material:	Huhn	Meersch. schw.	Hamm- mel	Mensch O	Mensch A	Mensch B
Rubiaceae:							
260. <i>Borreria laevis</i>	Blatt	(1280)	1280	1280	5120	160	640
261. <i>Coffea liberica</i>	Blatt	640	(640)	640	640	640	640
262. <i>Ixora coccinea</i>	Blatt	2560	1280	5120	10240	1280	1280
263. "	Blume	(5120)	2560	5120	10240	5120	5120
264. "	Frucht	20480	10240	5120	20480	20480	20480
265. <i>Ixora finlaysoniana</i>	Blatt	10240	5120	10240	(10240)	1280	2560
266. "	Blume	0	0	0	0	0	0
267. <i>Ixora laxiflora</i>	Blatt	5120	10240	5120	12800- -5120	640- -10240	320- -5120
268. <i>Ixora lutea</i>	Blatt	2560	10240	10240	320- 5120	160- -2560	320- -2560
269. <i>Ixora macrothyrsa</i>	Blatt	640	1280	640	160	(160)	320
270. "	Blume	1280	2560	640	160	320	320
271. <i>Ixora spec.</i>	Blatt	5120	(20480)	5120	640	5120	2560
272. "	Blume	80	160	80	80	80	40
273. <i>Morinda citrifolia</i>	Blatt	80	1280	2560	(1280)	640	640
274. <i>Oldenlandia herbacea</i>	Blatt	0	(40)	0	0	0	0
275. <i>Rondeletia odorata</i>	Blatt	5120	2560	5120	(2560)	(2560)	(1280)
276. "	Blume	10240	10240	2560	5120	2560	2560
Rutaceae:							
277. <i>Murraya exotica</i>	Blatt	0	0	0	0	0	0
Sapindaceae:							
278. <i>Nephelium litchi</i>	Blatt	1280	5120	1280	2560	10240	1280
Sapotaceae:							
279. <i>Achras sapota</i>	Blatt	5120	10240	5120	(10240)	2560	1280
Scrophulariaceae:							
280. <i>Angelonia salicariaefolia</i>	Blatt	(40)	80	80	(80)	160	80
Simarubaceae:							
281. <i>Quassia amara</i>	Blatt	2560	320	2560	(1280)	(5120)	320
Solanaceae:							
282. <i>Solanum macranthum</i>	Blatt	0	0	0	0	0	(40)
Sterculiaceae:							
283. <i>Guazuma ulmifolia</i>	Blatt	40	0	0	0	0	0
284. <i>Theobroma cacao</i>	Blatt	1280	160	(80)	40	0	40
Tiliaceae:							
285. <i>Corchorus capsularis</i>	Blatt	0	0	0	0	0	0
Verbenaceae:							
286. <i>Clerodendron fragrans</i>	Blatt	40	(160)	80	(160)	80	(80)
287. <i>Clerodendron speciosissimum</i>	Blatt	0	40	0	0	0	0
288. "	Blume	0	40	0	0	0	0
289. <i>Duranta erecta</i>	Blatt	320	320	320	640	640	640
290. "	Blume	160	320	160	320	320	320
291. "	Beere	160	160	160	320	160	160
292. <i>Cytharexylum spinosum</i>	Blatt	0	40	0	0	0	(40)
293. <i>Holmskiöldia sanguinea</i>	Blatt	0	40	0	0	0	(40)
294. "	Blume	0	40	0	40	0	(40)
295. <i>Lantana camara</i>	Blatt	0	0	0	0	0	0
296. "	Blume	0	0	0	0	0	0
297. "	Frucht	0	0	0	0	0	0
298. <i>Petrea arborea</i>	Blatt	0	0	0	0	0	0
299. <i>Stachytarpheta cayennensis</i>	Blatt	320	640	320	160	(640)	320
300. "	Blume	0	0	0	0	0	0
301. <i>Stachytarpheta jamaicensis</i>	Blatt	(160)	160	160	80	(320)	160
302. "	Blume	(80)	(160)	(80)	(40)	(160)	80
303. <i>Tectona grandis</i>	Blatt	40	40	0	40	40	40
Zingiberaceae:							
304. <i>Aframomum melegueta</i>	Blatt	40	40	0	80	40	40
305. <i>Curcuma xanthorrhiza</i>	Wurzel	160	320	80	320	160	80
306. <i>Renealmia exaltata</i>	Blatt	320	1280	320	640	1280	1280

Untersuchte Species:	Material:	Haemagglutination der Erythrocyten von					
		Huhn	Meer- schw.	Ham- mel	Mensch O	Mensch A	Mensch B
Farrenpflanzen:							
307. <i>Adiantum</i> spec.	Blatt	(2560)	160	10240	640	320	320
308. <i>Asplenium</i> spec.	Blatt	80	160	0	0	0	0
309. <i>Lygodium</i> spec.	Blatt	1280	2560	1280	640	640	640
310. <i>Marsilia</i> spec.	Blatt	0	0	0	0	0	0
311. <i>Nephrolepis</i> spec.	Blatt	40	0	(40)	0	0	0
312. <i>Pteris</i> spec.	Blatt	80	40	160	160	160	(160)
313. <i>Salvinia natans</i>	Blatt	0	0	0	0	0	0

(= 6%), 1 von 30 *Gramineae* (= 3%) und 0 von 42 *Euphorbiaceae* (= 0%).

Die restlichen 90 Extracte (= 28,8%) reagierten gegenüber den verschiedenen untersuchten Blutarten nicht gleichartig. So kam es beispielsweise bei 9 von den 303 Extracten teilweise zu Haemolyse und teilweise zu Haemagglutination (= 2,9%), bei 6 Extracten zeigte sich teils Lyse, teils keinerlei Beeinflussung (= 1,9%) und bei 8 Extracten teils Lyse, teils Haemagglutination und teils keine Beeinflussung (= 2,5%), abhängig von der Blutart.

Haemolyse fand sich demnach bei allen oder wenigstens einzelnen der untersuchten Blutarten in 45 von 313 Fällen (= 14,4%).

Durch verschiedene Extracte wurde gelegentlich nur eine einzelne der untersuchten Blutarten haemagglutiniert. So agglutinierten Extracte von *Trichanthera gigantea*, *Commelina nudiflora* und *Andropogon nardus* nur Hühnerblut, Extracte von *Jatropha podagrica* und *Cassia spectabilis* (II) nur Hammelblut, Extracte von *Erythrina glauca* und *Glycine maxima* nur Menschenblut O und Extracte von *Caryota mitis* und *Solanum macranthum* nur Menschenblut B. Ausschliesslich Meerschweinchenblut wurde durch 4 Extracte in einer Concentration von über 1/100 und durch 10 Extracte in einer Concentration unter 1/100 haemagglutiniert. Meerschweinchenblut erwies sich in vielen Fällen als besonders empfindlich für die Haemagglutination.

Auch das umgekehrte kam gelegentlich vor, dass nämlich eine Blutart nicht, die 5 anderen aber agglutiniert wurden.

Bei den hier untersuchten Extracten fanden sich in keinem Fall jene grossen Unterschiede in der Empfindlichkeit der verschiedenen menschlichen Blutgruppen, die BOYD bei *Phaseolus lunatus* (Extract aus den Bohnen) nachweisen konnte und die durch uns – wie durch alle anderen Nachuntersucher – bestätigt werden konnte.

Besonders hervorgehoben sei noch die haemagglutinierende Wir-

kung des Extractes aus dem Samen von *Canavalia ensiformis* gegenüber Meerschweinchenblut. Hier kam es bis zur Endconcentration von 1/20480–1/163840 zu starker Haemagglutination. Ebenso war die haemolysierende Wirkung des Extractes aus dem Blatt von *Enterolobium cyclocarpum* besonders stark, denn Meerschweinchenblut und Menschenblut B wurde bis zur Endconcentration von 1/163840 haemolysiert, Menschenblut O und A bis zur Endconcentration von 1/81920.

Eine Reihe von Extracten wurden 5–60 Minuten lang im Wasserbade erwärmt und hiernach erneut auf ihre haemagglutinierende bzw. haemolysierende Fähigkeit untersucht. Diese Versuche sind in der Tabelle 2 zusammengefasst. In der Regel blieb die Wirkung der Extracte unverändert. In einzelnen Fällen lief der Titer zurück, so beispielsweise bei *Tephrosia candida*, *Lochnera rosea*, *Malachra alciiformis* und sehr stark bei *Canavalia ensiformis*. Bei *Lantana camara* kam es nach Erwärmen zu einer leichten Haemagglutination bzw. Haemolyse, während der native Extract wirkungslos war.

Bei dem Extract von *Beloperona guttata* wurde der Einfluss verschieden hoher Temperaturen untersucht. Aus der Tabelle 3 geht hervor, dass Meerschweinchenblut durch den nativen Extract bis zur Endconcentration von 1/2560 haemagglutiniert wird, dass Erwärmen auf 56° und 80° hierauf keinen Einfluss hat, dass aber schon 5 Minuten langes Erhitzen auf 100° den Titer auf 1/40 sinken lässt.

Besonders interessant war ein Erwärmungsversuch mit dem Extract von *Strophantus spec.*, der in Tabelle 4 zusammengestellt ist. Der native Extract reagierte mit Hühnerblut in der Verdünnung von 1/40 gar nicht, nach Erwärmen auf 56° und 63° aber deutlich mit Haemagglutination und nach Erwärmen auf 70° mit Haemolyse in der Verdünnung von 1/40. Bei stärkerem Erwärmen nahm die Lyse zu, um nach 30 Minuten langem Erwärmen auf 100° in der Verdünnung von 1/640 wenigstens noch partiell zu lösen.

Z u s a m m e n f a s s u n g.

Von 313 Pflanzenextracten, meistens aus Blättern hergestellt, wurde im Reagensglasversuch die Wirkung auf Blut von Mensch O, A, B, Hammel, Meerschweinchen und Huhn festgestellt. Von den 313 Extracten waren 87 (= 27,8%) wirkungslos, 114 verursachten bei allen Blutarten Haemagglutination (= 36,4%) und 22 Haemolyse (= 7,0%). Die restlichen 90 Extracte (= 28,8%) reagierten

TABELLE 2

Einfluss des Erwärms auf die haemagglutinierende Wirkung

Species:		Erwärmungs- dauer 100° C. Min.	Nativ:			
			Huhn	Meerschw.	Hammel	O
Acanthaceae:						
<i>Asystasia gangetica</i>	Blatt	5	80	(160)	80	160
<i>Beloperona guttata</i>	Blatt	10	320	640		
"	Blume	10	(2560)	5120		
<i>Megas kepasma erythroclamyd</i>	Blatt	5	80	80	80	80
"	Deckblatt	5	0	160	0	40
Apocynaceae:						
<i>Allamanda cathartica</i>	Blatt	10		160H	160	160H
<i>Cryptostegia madagascariensis</i>	Frucht	10	40	320	(80)	0
<i>Lochnera rosea</i>	Blatt	10	0	320H	0	40
"	Blume	10	0	320H	40H	80H
<i>Strophantis spec.</i>	Blatt	10	40	40	80	1280
Combretaceae:						
<i>Combretum rotundifolium</i>	Blatt	10			5120	640
<i>Laguncularia racemosa</i>	Blatt	10	1280	320	1280	1280
<i>Terminalia catappa</i>	Blatt	10	(640)	320H	160	160
<i>Terminalia dichotoma</i>	Blatt	10	1280	5120	(2560)	(1280)
Leguminosae:						
<i>Caesalpinia coriacea</i>	Blatt	5	20480	10240	(640)	640
"	Blatt	60				
<i>Calliandra brevipes</i>	Blatt	10	2560	640	2560	2560
<i>Canavalia ensiformis</i>	Blatt	10	20480	20480	20480	20480
<i>Indigofera subulata</i>	Samen	10	(2560)	(2560)	(2560)	(2560)
"	Blatt	10	1280	160	1280	320
<i>Inga spec.</i>	Blatt	5	10240	20480	10240	20480
"	Blatt	60				
<i>Pithecolobium dulce</i>	Blatt	5	1280H	2560H	1280H	640H
"	Blatt	60				
<i>Sesbania grandifloris</i>	Blatt	5	320H	(20480)	320H	320H
"	Blatt	60				
<i>Sesbania sericea</i>	Blatt	60	640	2560	640	640
<i>Tephrosia candida</i>	Blatt	5	80	160	(160)	2560
<i>Tephrosia maxima</i>	Blatt	10	160	2560	2560	2560
Malvaceae:						
<i>Hibiscus sabdariffa</i>	Frucht	5	160H	320H	160H	80H
<i>Malachra alceifolia</i>	Blatt	5	(160)	80H	40	(320)
<i>Malachra radiata</i>	Blatt	5	(160)	80	80	1280
<i>Sida acuta</i>	Blatt	5	640	320	160	2560
<i>Sida glomerata</i>	Blatt	5	320	320	320	320
"	Samen	5	(80)	80	40	(40)
<i>Sida rhombifolia</i>	Blatt	5	(640)	80	160	
Rubiaceae:						
<i>Coffea liberica</i>	Blatt	10	640	(640)		
<i>Ixora finlaysoniana</i>	Blatt	10	10240	5120		
<i>Ixora laxiflora</i>	Blatt	10	5120	10240		
<i>Ixora lutea</i>	Blatt	10	2560	10240		
<i>Rondeletia odorata</i>	Blatt	10	5120	2560		
"	Blume	10	10240	10240		
Verbenaceae:						
<i>Duranta erecta</i>	Blatt	10	320H	320H	320H	640H
"	Blume	10	320H	160H	160H	320H
"	Beere	10	160H	160H	160H	320H
<i>Lantana camara</i>	Blatt	10	0	0	0	0
"	Blume	10	0	0	0	0
"	Frucht	10	0	0	0	0

niger Pflanzenextracte. Legende wie in Tabelle 1. H = Haemolyse.

A	B	Erwärmt auf 100°					
		Huhn	Meerschw.	Hammel	O	A	B
80	(80)	0	40	0	0	0	0
		0	40				
		0	0				
80	80	0	0	0	0	0	0
(160)	80	0	0	0	0	(40)	0
320H	160H		80	(80)	(80)	(80)	(80)
40	40	80	80	(160)	0	0	0
40	0	0	0	0	0	0	0
80H	80H	0	0	0	0	0	0
1280	1280	320H	320H	160H	320H	320H	320H
320	320			320	(320)	160	320
320	320	1280	1280	5120	1280	1280	640
160	160	160	320	320	320	160	320
160	(1280)	320	320	160	320	160	160
320	320	5120	5120	5120	320	320	(320)
		20480	20480	20480	5120	5120	1280
320	(1280)	640	1280	640	(2560)	160	640
2560	5120	0	1280	0	0	0	0
160	640	1280	20480	1280	320	160	160
160	320	(640)	640	640	320	(80)	160
2560		5120	10240	(2560)	5120	2560	
		20480	20480	5120	2560	5120	
640H	1280H	(640H)	(1280H)	640H	(320H)	320H	(320H)
		(640H)	2560 Aggl.	(320H)	(160H)	(1280H)	(640H)
			640 Lyse				
640H		80H	80H	80H	80H	80H	
		160H	160H	80H	160H	(320H)	
1280	2560	1280	2560	1280	160	2560	160
2560	2560	0	0	0	0	0	0
80	80	80	160	1280	640	80	80
80H	80H	80	(160H)	(80H)	80	80	40H
1280	(160)	0	0	0	0	(40)	0
5120	2560	(40)	(160)	80	(320)	(80)	(80)
0480)	2560	0	80	0	0	0	0
640	(640)	40	40	(80)	40	40	0
(80)	(80)	0	(40)	0	0	0	0
		0	320	160			
		1280	(640)				
		5120	10240				
		2560	10240				
		1280	10240				
		1280	2560				
		5120	5120				
640H	640H	320H	640H	320H	320H	(640H)	320H
320H	320H	160H	320H	160H	320H	320H	(320H)
						640 Aggl.	
160H	160H	320H	(640H)	160H	320H	320H	160H
0	0	0	80H	0	0	(40)	(80H)
0	0	640	40	40	80	80	80
0	0	80	80H	40	40	(80)	40

TABELLE 3.

Haemagglutination von Meerschweinchenblut durch erwärmten Extract von *Beloperona guttata*.

Erwärmen des Extractes auf:	Agglutination von Meerschweinchenblut:
nativ	$\frac{1}{2560}$
56°60'	$\frac{1}{5120}$
80°10'	$\frac{1}{1280}$
100°5'	$\frac{1}{40}$
100°10'	$\frac{1}{40}$ partiell

TABELLE 4.

Haemagglutination bzw. Haemolyse von Hühnerblut durch erwärmten Extract von *Strophantus spec.*

Erwärmen des Extractes auf:	Reaction mit Hühnerblut:
nativ	$\frac{1}{40}$ negativ
56°60'	$\frac{1}{40}$ haemagglutiniert
63°60'	$\frac{1}{40}$ haemagglutiniert
70°10'	$\frac{1}{40}$ partielle Lyse
80°10'	$\frac{1}{80}$ Lyse
	$\frac{1}{160}$ partielle Lyse
100°10'	$\frac{1}{320}$ Lyse
100°30'	$\frac{1}{320}$ Lyse
	$\frac{1}{640}$ partielle Lyse

den verschiedenen Blutarten gegenüber nicht gleichartig. Erwärmen der Extracte auf 100° hatte in den meisten Fällen keinen Einfluss auf die haemagglutinierende Fähigkeit.

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THE USE OF AN EGG AGAR MEDIUM WITH NEUTRAL RED FOR THE DIFFERENTIATION BETWEEN *M. TUBERCULOSIS* AND ACID-FAST SAPROPHYTES

by

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(Received April 21, 1956).

For many years, acid-fast saprophytes have complicated the identification of *M. tuberculosis*. Among others, PINNER (9) expressed the opinion that the problem was far from being solved. In the years after the first world war, several methods were described which made it easier to distinguish between the two groups of organisms.

Briefly, the following methods are used. In vitro tests only are discussed, mention of animal experiments being purposely avoided.

DUBOS and MIDDLEBROOK's studies (6) of the characteristic arrangement of pathogenic *M. tuberculosis*, and their belief that "cords" were a typical feature of these organisms are well known. This phenomenon had been described many years before by ROBERT KOCH. It is not usually observed with acid-fast saprophytes. It should be noted that pathogenic *M. tuberculosis* do not invariably form cords in DUBOS' medium, and that this phenomenon sometimes occurs with acid-fast saprophytes (5).

BLOCH (4) reported that pathogenic *M. tuberculosis* do not reduce methylene blue and other redox dyes, whereas apathogenic strains and acid-fast saprophytes do. DUBOS and MIDDLEBROOK (6) further showed that pathogenic *M. tuberculosis* stain with neutral red, whereas acid-fast saprophytes mostly give a negative reaction.

Acid-fast saprophytes are insensitive to isoniazid (3). MIDDLEBROOK (8) further proved that *M. tuberculosis* which are resistant to isoniazid produce a negative catalase test, contrary to sensitive *M. tuberculosis* and acid-fast saprophytes.

It has been known for a long time that pathogenic *M. tuberculosis* do not usually grow on broth agar and Löwenstein's medium at room temperature (approximately 20° C.). Conversely, acid-fast saprophytes usually do grow on these media at room temperature.

It may seem redundant to add yet another method to those known up to now. However, the simplicity and reliability of the following distinction between acid-fast saprophytes and human (and bovine) *M. tuberculosis* is, in my opinion, of importance in routine laboratory diagnosis.

0.025% neutral red and 0.02% norit is added to the so-called B medium (BEEUWKES (1)), which is made up as follows:

4	g	monopotassium phosphate
0.4	g	magnesium sulphate
1	g	magnesium citrate
6	g	asparagine
20	ml	glycerol
1	l	distilled water

Sterilize for 1 hour at 105° C. Adjust pH to 6.6, then add 1.5 g amylum per 100 ml of saline mixture and heat until gel formation takes place. Add 2 g agar per 100 ml of saline mixture. (Extract difco agar for 24 hours at room temperature with abundant acetone and dry at room temperature). Sterilize for 1 hour at 105° C. in 200 ml containers. Homogenize 2 egg yolks in a container with glass beads. Heat the salt-agar mixture and the egg yolks to 52° C. Now mix the contents of the two containers, and finally add 1.25 ml sterile aqueous 2% malachite green solution, 25 mg neutral red and 20 mg norit. The medium then shows a brownish colour.

Acid-fast saprophytes change the colour of this medium to light to dark violet; with human as well as bovine *M. tuberculosis* it remains a brown colour. The colour difference is easily observed by comparison with a non-inoculated control tube.

We observed this difference in 55 acid-fast saprophytes and 120 strains of *M. tuberculosis*. All the *M. tuberculosis* strains showed a light-red culture, sharply contrasting with the brown substratum.

The saprophytes could be divided into those causing an unmistakable violet coloration of the medium and those which produced a yellow pigment as well. Both groups produced acid in the agar medium.

M. tuberculosis do not produce acid. Since *M. tuberculosis* do not split amylum, no acid being produced in the described egg agar

medium, amylum seems a superfluous component of the medium described above. The hydrolysis of amylum by acid-fast saprophytes was described by GORDON and SMITH (7). They reported, among other things, that all of 71 strains affected amylum.

The following table might be used to differentiate between *M. tuberculosis* and acid-fast saprophytes.

	Cords	Redox dye	Neutral red	Isoniazid	Catalase reaction	B medium plus neutral red
<i>M. tuberculosis</i>	mostly positive	mostly negative	mostly positive	sensitive	positive	no acid production
<i>M. tuberculosis</i> (insensitive to isoniazid)	mostly positive	mostly negative	mostly positive	insensitive (10 γ positive)	negative	no acid production
Saprophytes	mostly negative	mostly positive	mostly negative	insensitive	positive	acid production, possible pigment production

Summary.

Differentiation between acid-fast saprophytes and *M. tuberculosis* is possible by the addition of neutral red to the B medium. Acid-fast saprophytes split amylum and produce a violet colour, whereas *M. tuberculosis* do not split amylum, so that the brown colour is maintained.

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SOME DATA ON THE CHARACTER OF STAPHYLOCOCCI SURVIVING THE ACTION OF PENICILLIN

by

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(Received December 20, 1955).

The replica method as described in our previous publication (1955) has evidenced that after a 6–10 hours' contact of *Staphylococcus aureus* with penicillin, part of the organisms may survive the action of this antibiotic. Since in spite of the presence of the antibiotic microcolonies appeared to be formed, we concluded that these survivors were not "resting bacteria". Less survivors were replicated after 6–10 hours from a primary plate containing a critical concentration of penicillin (i.e. the minimal lethal concentration) than from plates containing higher concentrations. In the present publication the character of these "survivors" is further investigated.

M a t e r i a l s a n d m e t h o d s were as described in the previous publication. In several experiments the number of colonies on the replica plate was compared with their frequency on nutrient agar media as produced by 0.05 ml of tenfold dilutions of a 24 hours' culture. These frequencies were indicated as 10^{-1} , 10^{-2} , $10^{-2'3}$ (in between 10^{-2} and 10^{-3}) or $\bar{1}$, $\bar{2}$, $\bar{2-3}$.

Experiment I. The ratio in which Staphylococci survive the action of penicillin has been studied. In Table 1 the fraction of the number of Staphylococci producing colonies on the replica plate is given.

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TABLE 1.

Relative quantity of survivors of *Staph. aureus* 1375 transferred after various periods of pre-incubation, from media containing different concentrations of penicillin.

Relative frequency of the colonies produced is estimated by comparison with number produced by 0.05 ml of known dilutions of a standard 24 h. broth culture incubated on plain agar for another 18 hours. $1 = 10^{-1}$, $1-2 =$ in between 10^{-1} and 10^{-2} .

Concentration of penicillin (U/ml)	Relative frequency of colonies on primary plate after 24 hours' incubation	Relative frequency of the colonies reproduced on replica-plate				
		Time of pre-incubation				
		2h	4h	6h	8h	12h
4/100	$\frac{1}{4}$	$\frac{1}{1}$	$\frac{1}{2-3}$	$\frac{2}{4}$	$\frac{1}{4}$	$\frac{1}{4}$
8/100	0	$\frac{1}{1}$	$\frac{2}{2}$	$\frac{4}{3}$	$\frac{1}{4}$	$\frac{4-5}{4-5}$
32/100	0	$\frac{1}{1}$	$\frac{2-3}{2}$	$\frac{2-3}{2}$	$\frac{1}{4}$	$\frac{4}{4}$
2	0	$\frac{1-2}{2}$	$\frac{2-3}{2}$	$\frac{2-3}{2}$	$\frac{1}{4}$	$\frac{4}{5}$
100	0	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{2}{2}$	$\frac{4-5}{4-5}$	

A tenfold difference is deemed significant. For *Staphylococcus aureus* strain 1375 used in the previous and present experiments the critical concentration is 8/100 U/ml. If replicas are made 4-6 hours after inoculation the rate of decrease is found to be higher for critical than for higher concentrations. The fraction of the number of staphylococci surviving the action of penicillin decreases steadily. If the contact on the primary plate lasts from 8-12 hours no difference is found between the fractions surviving on low and high concentrations.

Experiment II. Are survivors as sensitive to penicillin as the original strain? Bacteria pre-incubated on 8/100 U/ml and 1 U/ml were transferred by replicating to two parallel series of media, one containing 4/100 U/ml, the other no penicillin. After spreading of the inoculum on the surface of these media the cultures were incubated overnight. The results are presented in Table 2.

Apart from an insignificant exception all bacteria inoculated "directly" were found to be sensitive to 4/100 U/ml penicillin. The number of bacteria inoculated was found to be comparable to 10^{-4} and 10^{-5} dilutions of a 24 hours' broth culture (cf. second replica; 6th column). Other groups were replicated first on plain agar media, incubated for 7 hours, and then likewise transferred to and spread on the two parallel series. None of the bacteria pre-incubated on 1 U/ml were resistant to 4/100 U/ml. Two groups of bacteria originating on the 8/100 U/ml media could grow on the 4/100 U/ml media after 48 hours' incubation. One group developed 800 colonies, the other 500, both on a restricted area. Among untreated bacteria, giving rise to a comparable amount of colonies in the series without penicillin one group of a hundred bacteria developed as well colonies on the 4/100 U/ml media. In Table 2, second column, in between 10^{-5} and 10^{-6} (a $\frac{1}{300,000}$ part of a 24 h. culture) is found to be resistant to 8/100 U/ml if incubated during 2×24 hrs. The three groups described originate probably from this fraction. Apart from these exceptions all bacteria surviving the contact with penicillin were sensitive to 4/100 U/ml and are thus as sensitive as the original strain.

Experiment III. The rate of development was compared for colonies originating from survivors and for those from untreated bacteria (Table 3). Media were inspected at regular intervals after

TABLE 2.

Test of sensitivity of survivors of *Staph. aureus* 1375 transferred from media containing critical and medium concentrations of penicillin.

5 = comparable to 0.05 ml of a 10^{-5} dilution of a 24 h. broth culture plated on plain agar and incubated for another 18 hours.

Concentration of penicillin in primary medium (U/ml)	Relative frequency of colonies on primary medium after 2×24 h. incubation	Period of pre-incubation (hours)	Duration of incubation on intermediate plain agar medium (hours)	Frequency of the colonies	
				1st replica 4/100 U/ml. Number	2nd replica no penicillin. Ratio
8/100	5-6	6		0	5
		9		0	4-5
		10		3 colonies	4-5
1	0	6		0	4
		9		0	3
8/100	5-6	7	7	800 colonies	3
1		7	7	500 colonies	3
0		7	7	0	4
				100 colonies	3

replicating. The lapse of time necessary for producing visible colonies on a plain agar (secondary) medium was noted. We compared this rate for:

- A) cultures replicated from "primary" media containing critical, medium and high concentrations of penicillin, and
 - B) cultures and dilutions of cultures developing on plain agar "primary" media and replicated after the same periods of incubation as in A),
- with dilutions of an untreated 24 hours' culture plated directly on the secondary medium.

TABLE 3.

Rate of formation of visible colonies of *Staph. aureus* 1375 on secondary media replicated from A) media containing various concentrations of penicillin, B) plain agar primary medium, as compared with a corresponding amount of colonies originating from untreated bacteria that were not pre-incubated.

Numbers indicate interval in hours after the moment at which colonies inoculated directly on secondary medium became visible.

Pre-incubation (hours)	concentration of penicillin in primary medium			
	A			B.
	8/100 U/ml	2 U/ml	100 U/ml	—
0	—1	—1	0	0
1	—3	—1	+5	—2
3	0	+4	+5	—2
5½	+2½	+4	> +5	—4
8	—1	+5	+5	—5

Colonies originating from bacteria surviving high and medium concentrations of penicillin became visible only after a longer delay when compared with a corresponding amount of untreated staphylococci, either pre-incubated on the primary plain agar medium for the same period, or inoculated on the secondary medium at the moment of replication of the other series.

With both high and medium concentrations of penicillin the extent of this delay appeared to be positively correlated with the duration of pre-incubation. The period of contact necessary to bring about a pronounced delay was shorter for high than for medium concentrations.

With a critical concentration on the contrary, after a relatively short contact the development of the colonies on the replica-plates was found to be accelerated to some extent. After a contact of several hours with the latter concentrations, however, the development of colonies on the secondary plate was delayed.

This delay in the development of colonies has been demonstrated in yet another way. Every 3 hours colonies first visible on the replica-plate were marked with Indian ink on the bottom of the Petri dish. Table 4 illustrates the rate of increase of the number of colonies. Those originating from untreated bacteria became visible with much less variation than colonies developed from bacteria surviving the action of medium and high concentrations. Moreover the majority of the colonies of untreated bacteria appeared to develop in the "leading group"; colonies originating from survivors, however, are distributed over several groups, the first groups often being smaller than the following.

The extent of this variation appears to be correlated with the duration of the contact with the antibiotic, short contact with critical concentrations having no influence on the rate of development. After $2\frac{1}{2}$ hours' pre-incubation, however, the variation was found to increase, beyond 4 hours' contact the spreading was similar to the "high concentration" type.

Experiment IV. As reported in our previous publication we had succeeded with 8 consecutive replicas to produce the same constellation of "daughter colonies" on 5, 6, 7 plates. So we concluded that some of the organisms must have formed microcolonies on the medium containing 8/100 U/ml after 6-8 hours. This multiplication might have been either the result of the brief stimulation of growth by critical concentrations or it might be inherent to the action of any concentration of the antibiotic. The following experiment was carried out.

On media *a*) with penicillin in various concentrations, *b*) on plain agar, pre-incubated for various intervals, six consecutive replicas were made, using a fresh disc for each replica. The results are reported in Table 5.

TABLE 4.
Variation of growth rate of colonies of *Staphylococcus aureus* 1375 on replica-plates.
Number of colonies first visible in readings after various intervals.

Period of pre-incubation (hours)	Concen- tration of penicillin U/ml	Inoculum (dilution of 24 h culture)	Number of hours after replicating										
			8	9	12	14	17	20	24	33	44	66	
0 (direct inoculation)		10 ⁻⁵		421	+ 28	+ 24			+ 6		+ 2		
1 (replica)	8/100 1	10 ⁻⁴		284	+ 17	+ 19	+ 8	+ 3			+ 3	+ 1	
		10 ⁻³		538	+ 315	+ 207	+ 58	+ 5	+ 18		+ 18		
	50 —	10 ⁻³		545	+ 121	+ 186	+ 22	+ 8	+ 5	+ 24	+ 81		
		10 ⁻⁴		398	+ 7	+ 5	+ 9	+ 2	+ 1	+ 20			
2½ (replica)	8/100 1	10 ⁻³		204	+ 85	+ 77	+ 17	+ 23	+ 2	+ 4			
		10 ⁻³			99	+ 121	+ 44		+ 13	+ 9			
	50 —	10 ⁻²			276	+ 301	+ 129	+ 20	+ 64	+ 48	+ 5		
		10 ⁻⁴		426	+ 9	+ 24	+ 3		+ 1	+ 1	+ 1		
4 (replica)	8/100 1	10 ⁻³		79	+ 92	+ 57	+ 38	+ 13	+ 6	+ 8	+ 1	+ 2	
		10 ⁻²			+ 171	+ 264	+ 224	+ 66	+ 24	+ 39	+ 2	+ 5	
	50 —	10 ⁻²										+ 5	
						310	+ 78	+ 107	+ 34	+ 55	+ 4	+ 5	
6 (replica)	8/100 1	10 ⁻²	132	+ 136	+ 187	+ 177	+ 143		+ 31	+ 38	+ 2		
		10 ⁻²		109	+ 182	+ 204	+ 114	+ 50	+ 25	+ 43	+ 2	+ 3	
	50 —	10 ⁻¹		44	+ 354	+ 342	+ 187	+ 147	+ 33	+ 59	+ 16		
		10 ⁻⁵	390	+ 25	+ 5	+ 6							
8 (replica)	8/100 1	10 ⁻²		171	+ 92	+ 245	+ 55	+ 38	+ 30	+ 49	+ 5	+ 6	
		10 ⁻¹			291	+ 569	+ 337	+ 150	+ 75	+ 229	+ 18	+ 11	
	50 —	10 ⁻¹			401	+ 541	+ 293	+ 270	+ 62	+ 126	+ 37		
		10 ⁻⁵	286	+ 39	+ 13								

TABLE 5.
Relative frequency of the colonies of *Staph. aureus* 1375 estimated on six consecutive replica-plates.

Primary media contained various concentrations of penicillin, 2, 3, 3-4 etc. corresponding to relative frequency of the colonies produced on plain agar by 0.05 ml of tenfold dilutions of a 24 h. culture. k = number of colonies counted.

duration of pre-incubation (hours)	0		1		3		5		7½		10½	
concentration of penicillin in primary medium (U/ml)	8/100	50	8/100	50	8/100	50	8/100	50	8/100	50	8/100	50
1	2	2	2	3	2-3	3-4	1520k	372k	46k	169k	7k	70k
2	2-3	3	2-3	3-4	2-3	3-4	900k	548k	35k	352k	6k	78k
3	3	3	3	3-4	3	3-4	680k	740k	15k	184k	2k	54k
4	3-4	3-4	3	4	3	3-4	332k	592k	12k	200k	4k	42k
5	3-4	3-4	3-4	3-4	3-4	3-4	204k	284k	11k	159k	—	28k
6	4	4	3-4	4	3-4	3-4	159k	132k	6k	131k	—	16k

From these data we calculated the ratio:

$$\frac{\text{number of colonies on 6th replica}}{\text{number of colonies on 1st replica}}$$

These ratios were:

- 1 : 30 for untreated bacteria pre-incubated 1—3 hrs.
- 1 : 10 for untreated bacteria pre-incubated 5 hrs.
- 1 : 30 for bacteria pre-incubated for 1 hr on 8/100 U/ml medium.
- 1 : 10 for bacteria pre-incubated for 3—7 hrs on 8/100 U/ml medium.
- 1 : 10 for bacteria pre-incubated for 1 hr on high concentration.
- 1 : 3 to 1 : 1 for bacteria pre-incubated for 3—7 hrs on high concentration.

These results apparently give additional evidence that the surviving bacteria must have formed microcolonies in the presence of the antibiotic. This multiplication sets in at an earlier moment and is more pronounced for high concentrations. We repeatedly made the following observations:

1) on replicas, made from media containing high concentrations of penicillin the first plate often had a smaller number of colonies than the following. First replicas from media with 8/100 U/ml or plain agar media always bore more colonies than the following.

2) Colonies originating from "survivors" of high concentrations of penicillin varied markedly in size (up to 6 mm in diameter).

Experiments III and IV were moreover carried out with *E. coli*, *Proteus mirabilis* and *Haemophilus influenzae*. Among these bacteria as well, a small number appeared able to survive the action of high concentrations of penicillin. Table 6 shows a delay in the development of colonies of the penicillin-treated *E. coli* 9002 and K12 comparable with the retardation observed with *Staph. aureus* 1375. The rate of variation was found to have increased, but the change was not as striking as for *Staph. aureus*.

TABLE 6.

Number of extra hours necessary to develop visible colonies originating from penicillin-treated *E. coli* as compared with colonies grown from untreated bacteria (0 hours).

<i>E. coli</i> strain	concentration of penicillin in primary medium	duration of pre-incubation		
		3h	6h	9h
9002	500 U/ml	+1	+2	+2
K12	500 U/ml	+1	+5	>+5

Experiment IV such as reported in table 4, however, failed to give clear results with *E. coli* 9002 and K12 and *Proteus mirabilis*. For media with or without penicillin and for various periods of pre-incubation no difference was found in the ratio of the number of daughter-colonies on the first and the sixth replica-plates. It was observed, however, that *Proteus mirabilis*, after pre-incubation for 12 hours on media containing 10% horse-serum and 500 U of penicillin produced on several consecutive replica-plates daughter-colonies showing a strikingly similar constellation.

TABLE 7.

Relative frequency of the colonies of α - and β -Streptococci estimated on six consecutive replica-plates.

Primary media contained 50 U/ml of penicillin. For legend see table 4.

duration of pre-incubation (hours)		0	1	3	5	8	14½
<i>Streptococcus</i> 5013 (β -haemolytic)							
replica-plates	1	2	2-3	620k	400k	43k	1k
	2	2	2-3	420k	200k	45k	7k
	3	2-3	3	340k	140k	20k	13k
	4	2-3	3-4	188k	57k	10k	3k
	5	3	4-5	200k	29k	7k	3k
	6	3	4-5	100k	28k	7k	0
<i>Streptococcus</i> 4879 (α -haemolytic)							
replica-plates	1	1-2	2-3	3	3	3	4
	2	2	3	3-4	3	3-4	4
	3	2	3	3-4	3-4	3-4	4-5
	4	2-3	3-4	3-4	3-4	3-4	5
	5	3	3-4	4	4	4	5
	6	3	4	4	4	4	5

This same experiment was repeated for α - and β -Streptococci (Table 7). For up to 8 hours' pre-incubation no significant change was observed in the ratio:

$$\frac{\text{number of colonies on first replica}}{\text{number of colonies on sixth replica}}$$

The "paradoxical" increase of colonies on the second and the third replica was observed only in the last series of *Streptococcus* 5013. The number of colonies was too small to permit conclusions.

DISCUSSION.

For a critical evaluation of our observations it is necessary to connect them with the results of experiments published by others. The delay in the development of the colonies was evidenced by readings of the replica-plates at regular intervals (Table 3). We considered three possible explanations:

a) The growth of staphylococci is retarded by penicillin.

Retardation of growth has not been described up till the present. CHAIN and DUTHIE (1945) on the contrary, stated that penicillin does not affect the growth rate. Critical concentrations furthermore, seem to accelerate the growth during a short period. It does not seem very plausible that high concentrations of penicillin should retard the growth of a mere small fraction. Moreover, we have stated in our previous communication (1955) that penicillin does kill these survivors in combination with sub-inhibitory concentrations of streptomycin. Retardation of growth will neither benefit the action of streptomycin alone nor of streptomycin and penicillin together.

b) The survivors are bacteria that grow slowly and for that reason were not killed by penicillin. On the replica-plate again they grow more slowly than the majority of normal – untreated – bacteria.

If the slowly growing bacteria are “screened” by penicillin, this will result in increase of the retardation of the development of the colonies – the slowest being the most persistent. The variation of the growth rate, however, cannot be expected to increase, and the majority of the “screened” bacteria would be likely to develop at an equal – retarded – rate, a fraction being still more backward.

c) Penicillin modifies the bacteria, the delay is a lag-time needed for regaining the original structure.

Two different kinds of modifications should be distinguished:

1) Penicillin is bound irreversibly to an important cellular constituent. It depends on the rate of regaining the essential metabolites whether the balance can be restored. This theory is supported by PARKER (1953). EAGLE and MUSSELMAN (1949) have attributed the “bacteriostatic action” to a toxic effect of penicillin. From this effect the bacteria are considered to recover slowly.

According to these theories, the more firmly bound penicillin could be expected to be the more bactericidal. In our experiments however, critical concentrations effected less important changes than higher ones, if the duration of the lag-time is considered, but

their lethal effect is found to be stronger during the first hours of contact. Moreover the increase of multiplication on the primary medium along with higher concentrations and prolonged pre-incubation (cf. Table 4), cannot be explained by these hypotheses.

2) Penicillin effects a reversible, morphological change, the stronger as the contact is prolonged or the concentration of penicillin increased. As a result the lag-time is extended and the recovery is more complicated.

Morphological changes were for the first time described in 1940 by GARDNER. For full references see "Antibiotics" (FLOREY *et al.* 1949).

More recently two kinds of changes were described, the "large bodies" and the "L-forms". There is a consensus of opinion [DIENES (1947, 1948, 1949, 1953), TULASNE (1948, 1950), GRASSET and BONIFAS (1955), VON PRITZWITZ (1953)] that both large bodies and L-forms can return to normally shaped bacteria. L-forms and other reversible morphological changes effected by penicillin have been observed in several bacterial species: *Proteus*, *Haemophilus influenzae* (DIENES, 1947), *Salmonella typhimurium* (CARRÈRE and ROUX, 1954), *Streptococcus* (DIENES, 1953, SHARP, 1954). According to PULVERTAFT (1953) *Staph. aureus* up till then had never produced L-forms. On the other hand, among the bacteria mentioned none has ever been found to show a normal cellular division in the presence of high concentrations of penicillin.

Prof. TULASNE most kindly consented to investigate our *Staphylococcus* strain 1375. He did not succeed in obtaining structures corresponding to L-forms. He did observe "small colonies consisting of slightly hypertrophied elements of the *Staphylococcus* type" ¹⁾.

L-colonies in *Proteus*, appear to develop in the agar. In our experiments after pre-incubation on high concentrations of penicillin, less colonies developed on the first replica than on the second, third and fourth. It may be necessary to press repeatedly in order to catch the microcolonies, or to blast the large bodies.

As long as others emphatically deny the existence of L-cycles in *Staphylococcus*, and as long as we have not seen these forms ourselves, we can only hypothesize the existence of (dwarf) involution forms. This is based on the following observations:

1) The delay in the development of the colonies being much more

¹⁾ Dr DIENES informed me that he did observe L-colonies of *Staph. aureus* (DIENES, L. and SHARP, J. T. 1956. J. Bact. **71**, 208).

extensive after contact with higher than with critical concentrations, this may point to important structural changes.

2) As the increase in organisms is far more rapid than in normal growth, desintegration is more likely than growth.

3) According to circumstantial evidence the large bodies and the dwarf forms might be bedded in the agar, just like L-forms.

We suppose that critical concentrations mainly effect intermediary forms. In the discussion of EAGLE's zone phenomenon in our previous publication it was shown that a fraction of the survivors was killed by critical concentrations only after a very long delay. These persistent survivors might be developed involution forms.

Communications on the stimulation of growth by low concentrations are to be found in "Antibiotics" (1949) and in INGRAMS publication (1951). As another effect of these low concentrations is supposed to consist in preventing most of the intermediary forms to pass into dwarf forms, there may be a connection between the two actions.

We obtained evidence of the existence of micro-colonies formed by *Proteus mirabilis* on media containing up to a tenfold the high concentrations of penicillin and 10% horse-serum. The relative number of colonies was smaller than with *Staph. aureus* 1375.

A delay in the formation of colonies and an increased variation in the growth-rate were observed for two strains of *E.coli*. No evidence of a multiplication on the primary medium was obtained for these strains. This might indicate that the change of structure is not as radical as for *Staph. aureus*.

S u m m a r y.

1) The development of colonies originating from staphylococci surviving the influence of high and medium concentrations of penicillin, experiences a delay and the variation of their growth-rate increases as compared with untreated bacteria. Critical concentrations of penicillin have a brief growth-stimulating effect.

2) This delay in the development of colonies is connected with structural changes effected by penicillin. As the duration of the contact is longer and the concentrations higher, the changes become more radical.

3) The first degree of involution is thought to be an intermediary

form; next the "large body"; the more drastically transformed structures are supposed to be desintegration products of the large bodies.

4) With *Proteus mirabilis*, *E. coli*, *Streptococcus* and *H. influenzae* also, organisms surviving the action of lethal concentrations of penicillin were found. The structural changes in these organisms are, if comparable at all, less frequent and mostly less radical than those found in *Staphylococcus*.

Acknowledgment.

Our thanks are due to Prof. R. TULASNE, Strassburg, who took the trouble to investigate our strain 1375 and to Prof. Dr J. E. DINGER for his critical advice.

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THE YEAST *KLUYVEROMYCES AFRICANUS*
NOV. SPEC.
AND ITS PHYLOGENETIC SIGNIFICANCE

by

J. P. VAN DER WALT

(Received March 7, 1956).

The occurrence of fermentative yeasts with more than 8 ascospores per ascus had, until the isolation of *Kluyveromyces polysporus* (VAN DER WALT, 1956), been extremely rare. JÖRGENSEN (1909, 1940) described such a species, *Saccharomyces multisporus*, which had been isolated by HOLM from an English top yeast. Unfortunately, subcultures of this organism were apparently not maintained since neither STELLING-DEKKER (1931) nor LODDER and KREGER-VAN RIJ (1952) described this species in the monographs. *Saccharomyces multisporus* Jörgensen fermented glucose, saccharose and maltose and formed asci containing as many as eleven highly refringent spores. However, the ability to sporulate was quickly lost. Recently another fermentative budding species which forms asci containing up to 16 ascospores was isolated from soil. The existence of such yeast forms is thus probably more common than previously thought.

The following description of this new yeast is based on the standard methods of LODDER and KREGER-VAN RIJ (1952) for the determination of yeasts.

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C. the cells are oval to long oval $(1.8 - 6.9) \times (3.6 - 8.4) \mu$, single or in pairs. A few conjugated pairs measuring up to $(6.8 \times 13.8) \mu$ are present. After 1 month at 17°C. a sediment and ring are formed.

Growth on malt agar: After 3 days at 25°C. the cells

are oval to long-oval $(1.9 - 6.3) \times (2.5 - 7.5) \mu$, single or in pairs. After 1 month the streak culture is cream coloured, smooth and shiny.

Slide culture: No pseudomycelium is formed. A few chains of cells may be formed.

Sporulation¹⁾: An isogamous conjugation may or may not precede ascus formation. The spores are long oval to slightly reniform, often with an oil drop inside; 1-16 per ascus²⁾ (Figs. 1, 2 and 3). The spores are soon liberated.

Fermentation:	glucose	+	maltose	—
	galactose	+	lactose	—
	saccharose	—		
Sugar assimilation:	glucose	+	maltose	—
	galactose	+	lactose	—
	saccharose	—		

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: No growth.

Splitting of arbutin: Absent.

DISCUSSION.

To establish the taxonomic position of this species, it is necessary to decide what significance may be attached to its ascospore number with a maximum of sixteen.

It does not seem feasible to regard this organism as multispored since MARTIN (1954) only considers species of the *Endomycetales* with more than sixteen spores per ascus as multispored. Furthermore, the maximum spore number for the strain under discussion is constant — a feature lacking in the accepted multispored genera such as *Ascoidea* and *Dipodascus*. For these reasons the new species cannot be readily classified as a species of the multispored yeast genus, *Kluyveromyces*.

On the other hand, the differences between the species under discussion and those of the genera of the *Endomycetaceae* (*in sensu* Lodder et Kreger-van Rij) are sufficient to exclude it from any of these genera.

In the light of their recent hybridisation studies WICKERHAM

¹⁾ Spores were observed on Gorodkova agar and on the common sporulation media.

²⁾ The spores are acidoresistant-acidophilic on staining and were also observed to germinate.

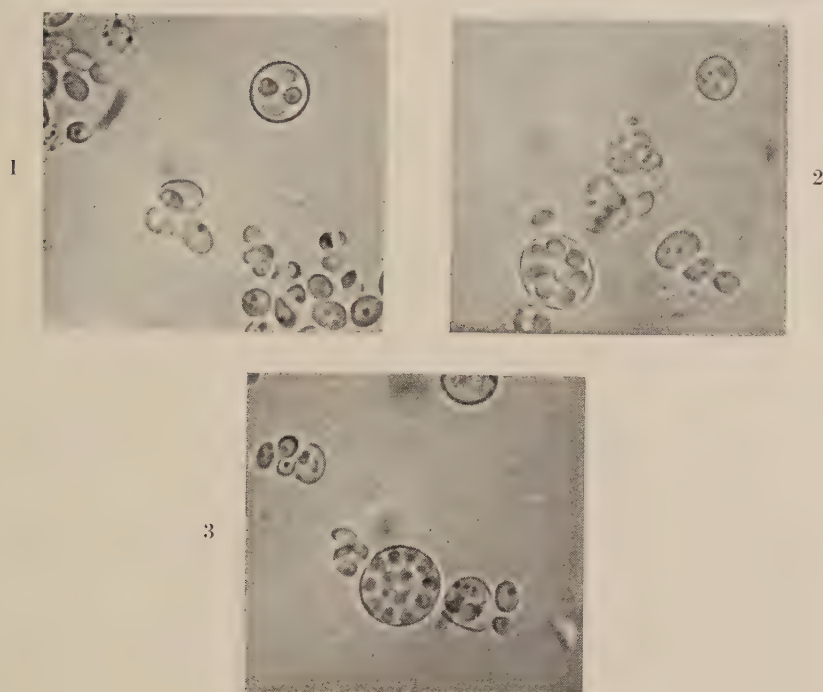


Fig. 1. *Kluyveromyces africanus*. Spores on Gorodkowa agar after 7 days.

Asci with 1 and 4 spores per ascus. $\times 1,000$.

Fig. 2. *Kluyveromyces africanus*. Spores on Gorodkowa agar after 7 days.

Ascus with 8 spores. $\times 1,000$.

Fig. 3. *Kluyveromyces africanus*. Spores on Gorodkowa agar after 7 days.

Ascus with 16 spores (15 spores in focus). $\times 1,000$.

and BURTON (1956 *a* and *b*), however, came to the conclusion that many species at present classified in the genus *Saccharomyces* constitute a genetically distinct group. Basing their differentiation not only on genetical but also on morphological and biochemical characteristics, they proposed to gather these related species into a new genus *Dekkeromyces*. The most characteristic feature of the genus *Dekkeromyces* is that in the one line of development the species form spheroidal ascospores, while in the other line the ascospores are "crescent-shaped". The presence of long oval to reniform ascospores in a budding, fermentative yeast not assimilating nitrate, forming 1—4 spores per ascus, would be indicative to classify the organism as a *Dekkeromyces* species. According to WICKERHAM and BURTON (1956 *a*), this could then be established by hybridisation experi-

ments, since most, if not all, *Dekkeromyces* species readily hybridise with one another.

Comparing the characteristics of the yeast from soil described above with those given by WICKERHAM and BURTON (1956 *a*), it immediately becomes evident that it possesses two of the criteria of the proposed genus, *viz.* the reniform shape of the ascospores and that these are very soon liberated on the sporulation medium. However, all the hitherto described *Saccharomyces* species with reniform ascospores, such as *Sacch. fragilis*, *Sacch. marxianus*, *Zygosacch. ashbyi*, *Sacch. dobzhanskii*, *Sacch. phaselosporus*, *Sacch. drosophilae* and *Sacch. delphensis*, as well as those species with spheroidal ascospores, *e.g.* *Sacch. lactis*, never form more than four spores per ascus. For this reason, the sixteen-spored species would be an exception, if grouped with the species.

On comparing this sixteen-spored species with *Kluyveromyces polysporus*, similar difficulties arise, despite the similarity of their ascospore shape. Although this species produces more than the usual number of ascospores per ascus, its maximum number is fixed and cannot be regarded as truly multispored. Furthermore, its asci never assume the large proportions noted for *Kluyveromyces polysporus*.

Hence, it must be accepted that this sixteen-spored species forms a clear intermediate between the typical multispored genus *Kluyveromyces* on the one hand, and the proposed *Dekkeromyces* with its one to four-spored species, on the other. The classification of this sixteen-spored species as either a *Kluyveromyces* or *Dekkeromyces* species, or perhaps in an intermediate genus, will depend on its sexual characteristics — the ratio of haploid to diploid cells in a vegetating strain and its ability to hybridise with *Kluyveromyces polysporus* and members of the proposed genus *Dekkeromyces*. Until such information becomes available, the species will provisionally be retained in the genus *Kluyveromyces*. It is therefore proposed to name the species *Kluyveromyces africanus*, for the continent of its isolation.

On the grounds of the taxonomic significance of the shape of the ascospore, VAN DER WALT (1956) established the phylogenetic relationship of the yeast genus *Kluyveromyces* with *Dipodascus uninucleatus*, since both these multispored genera of the *Endomycetales* form long oval to reniform ascospores. Accordingly, *Kluyveromyces* must be regarded as the most primitive yeast form yet known,

since it has not only retained the multisporeogenous character, but also the shape of the ascospores of the ancestral mould form, *Dipodascus uninucleatus*.

The derivation of *Kluyveromyces polysporus* from *Dipodascus uninucleatus* is in agreement with the views of many earlier investigators, such as ATKINSON (1915), GUILLIERMOND (1928, 1935, 1936), BIGGS (1937) and others, who regarded the genus *Dipodascus* as the progenitor of the ascosporeogenous yeasts. The stepwise transition of the ascospore number from multispored to one-four in the phylogenetic line, *Dipodascus uninucleatus* \rightarrow *Kluyveromyces polysporus* \rightarrow *Kluyveromyces africanus* \rightarrow *Dekkeromyces* species supports the classical conception in so far that *Dipodascus* is maintained as progenitor of the sporeogenous yeasts. Otherwise it challenges GUILLIERMOND's (1912) views in that it is no longer possible to regard all one to four-spored *Saccharomyces* species as derived from *Eremascus fertilis* via the hypothetical *Endomyces a.*

Kluyveromyces africanus nov. spec.

In musto maltato cellulae ovidiae aut longovidiae $(1.8-6.9) \times (3.6-8.4) \mu$, singulae aut binae. Cellulae conjugatae praesentes proprie $(6.8-13.8) \mu$ metiuntur. Sedimentum et annulus formantur.

In agar maltato cellulae ovidiae aut longovidiae $(1.9-6.3) \times (2.5-7.5) \mu$, singulae aut binae.

Cultura (post unum mensem, 17°C) albiflava, glabra, nitida.

Pseudomycelium nullum.

Copulatio cellularum aequarum ascorum plerumque conformationi praecedit. Asci etiam sine copulatione praecedente conformantur.

Ascosporae glabrae, longovidiae aut reniformae, fortasse globulos olei continent, 1—16 inasco. Ascosporae ex ascis celeriter liberantur.

Fermentatio glucosi et galactosi. In medio minerali cum glucoso et galactoso crescit. Nitras kalicus non assimilatur. In medio minerali cum alcohole aethylico non crescit. Arbutinum non finditur.

S u m m a r y.

A new budding yeast species isolated from soil is described. Its outstanding features are, firstly, the formation of asci containing up to sixteen long oval to reniform ascospores and, secondly, a

fermentative as well as oxidative metabolism. The assimilation of nitrate is absent and no pseudomycelium is formed.

The taxonomic position of the yeast is discussed and it is pointed out that, due to its exceptional ascospore number (1—16), it cannot be classified in any of the existing fermentative genera of the *Endomycetaceae* (*in sensu* Lodder et Kreger-van Rij). The reniform shape of its ascospores indicates, however, its close relationship with the multisporous genus *Kluyveromyces*, on the one hand, and the newly proposed one to four-spored genus *Dekkero- myces* on the other. The species is provisionally classified as a *Kluyveromyces* species, *Kluyveromyces africanus* nov. spec., until further information regarding its sexual characteristics becomes available.

By virtue of its more or less intermediate ascospore number, it establishes the direct derivation of the genus *Dekkero- myces* from *Dipodascus uninucleatus* via the multisporous yeast genus *Kluy- veromyces*.

Acknowledgement.

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AN ELECTRON MICROSCOPE STUDY ON THE SHAPE OF THE SPORES OF *BACILLUS POLYMYXA*

by

A. VAN DEN HOOFF and SIETSKE ANINGA

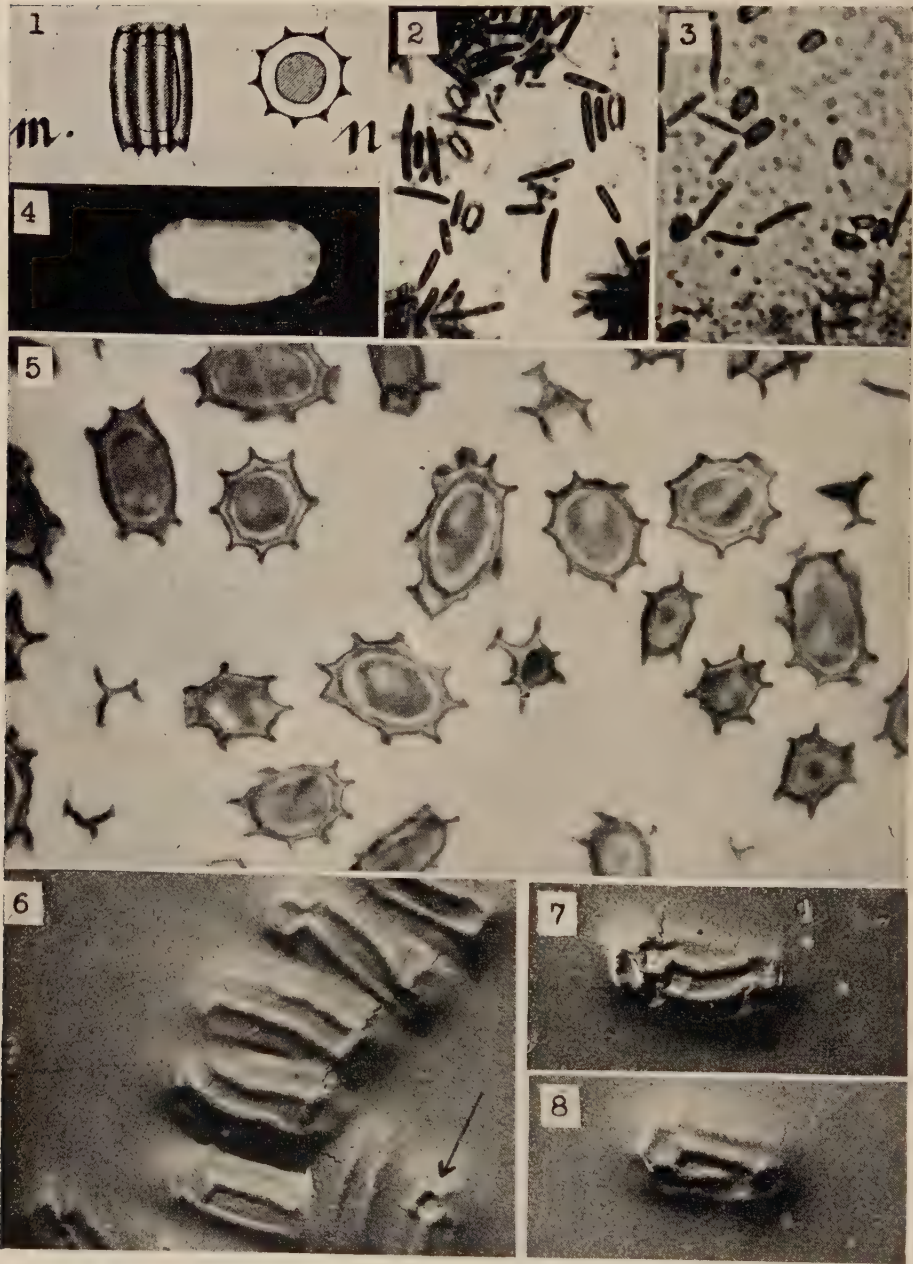
(Received May 5, 1956).

As early as 1897 A. MEYER described a peculiar structure specific for the spores of *Astasia asterosporus* syn. *Bac. polymyxa* (Prazmowski). His drawing (fig. 1) shows a "barrel" with longitudinal ribs and its star-shaped cross-section. As we never were able to confirm this observation by ordinary microscopy (see figs. 2 and 3), it seemed worthwhile to study the spores of *Bac. polymyxa* with the electron microscope.

The spore material was harvested from a six days old culture on potato-agar.

The direct study of the spores on formvar membranes was disappointing, because the spores were too opaque to show any surface detail. Only at the rim some points were visible just as in direct light microscopy (compare figs. 2 and 3 with fig. 4). So it seemed indicated to make ultra-thin sections by means of our Sjöstrand ultramicrotome. The spores were fixed in a 1% OsO₄-solution, buffered according to PALADE (1952) and were washed in four changes of physiological salt-solution; this thorough washing was necessary for removing adhering slimy material. After dehydrating in an alcohol series the spores were embedded in a mixture of 1 part methylmethacrylate and 19 parts butylmethacrylate. In order to get a dense suspension for sectioning the gelatin capsules, containing the spores suspended in the monomere with added catalyzer, were centrifuged to bring the spores down to the bottom.

As shown by figure 5 the sections of the spores indeed have the shape of a star as described by MEYER.



The photograph shows cross-, lengthwise- and tangential sections through the spores. From these sections can be deduced, that the spores have a cylindrical shape with rounded ends. On the outer surface we find more or less parallel ridges to the number of eight, sometimes seven. The ridges are very regular in shape, triangular in cross-section and evenly spaced.

It was however rather difficult to reconstruct from these sections the true shape of the spores. To solve this problem we tried to make replica's of the surfaces of the spores. To accomplish this it was necessary to prevent the spores from sticking to the replica-film and therefore the following technique was applied.

An object-glass was covered with a very thin film of gelatin in a way similar to the making of a bloodsmear. A suspension of the spores in water was brought on this film at a temperature of 50°C. The gelatin swelled a bit in the warm water and so the spores could sink in it a little. After drying the embedded spores were shadowed and then covered with parlodion. The replica was detached by immersion in warm water, taking the shadow material with it.

The results are shown by the figs. 6, 7 and 8. At the poles of the spore the ridges are no longer arranged parallel, but are forming a kind of honeycomb structure, enclosing one or more polygonal impressions (see arrow fig. 6). Curious are the many irregularities in the spores. It is evident, that these aberrations are not caused by shrinkage; MEYER observed that the appearance of the spore was not changed after drying and reswelling, so that its structure must be rather firm.

The picture of the sectioned spores agrees very well with the imprints of the spores in the parlodion film. As this study completely confirms the remarkable observations by MEYER, it must be concluded, that MEYER was an unusually keen observer.

In fig. 5 we can see the interior of the spores. The spore coat consists of an outer and an inner layer, separated by a regular space;

Fig. 1. Drawing of spore of *Bac. asterosporus* by A. MEYER, published in his "Practicum der botanischen Bakterienkunde", Jena 1903.

Fig. 2, 3. Spores of *Bac. polymyxa*, lightmicroscopical; 2000 \times .

Fig. 4. Electron micrograph of whole spore of *Bac. polymyxa*; shadowed; 12000 \times .

Fig. 5. Electron micrograph of ultrathin section of spores of *Bac. polymyxa*; 12000 \times .

Fig. 6, 7, 8. Replica's of spores of *Bac. polymyxa*; shadowed; 12000 \times .

the inner layer faintly follows the surface relief. We propose to apply the old terms exine and intine, also used by KNAYSI (1951), to the outer and inner coat of the spore capsule respectively. We realize, that what we call the intine cannot be identical with the intine as described by MEYER, because MEYER could not have been able to see such fine structures. The name intine however is in agreement with MEYER's definition.

We have got the impression, that the coat of the spore of *Bacillus polymyxa* is more rigid than the coats of other spores described in literature. In the center we see a core, separated from the intine by a regular, non-osmophilic space. In most cores we can observe a peripheral non-osmophilic spot, probably corresponding with the nuclear material described by ROBINOW (1953).

We have the intention to investigate the spores of the other members of the genera *Bacillus* and *Clostridium* in the same way.

A c k n o w l e d g e m e n t.

The authors wish to express their thanks to Prof. T. Y. KINGMA BOLTJES for suggesting the subject and for his advice, and Mrs. S. W. RAMP for making the sections.

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A SEARCH FOR *CRYPTOCOCCUS NEOFORMANS* IN MILK

by

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(Received July 3, 1956).

The first report of pathogenic yeasts in milk seems to be that of KLEIN (4). He isolated what would appear to be *Cryptococcus neoformans* (Sanfelice) Guillemon whilst searching for tubercle bacteria in the London milk supply. A guinea pig injected subcutaneously with sediment from a milk sample was found on autopsy to have developed a tumour of the groin which contained many spherical to oval yeast cells. Cultures from the tumour gave growth of a capsulated yeast which did not ferment sugars.

CARTER and YOUNG (1) in Glasgow reported that they had found *C. neoformans* in milk by the same technique. In the course of routine examination for tubercle, the centrifuged deposit from 50 ml of a bulked milk sample was injected into a guinea pig. The pig died 41 days later, and on autopsy was found to be suffering from a systemic yeast infection. On culture the yeast produced creamy mucoid colonies of budding cells, but no mycelium. It did not ferment sugars, and showed a capricious pathogenicity for mice and for other guinea pigs into which it was injected. CARTER and YOUNG examined the herd from which the milk sample had come but found no obvious signs of disease.

Cryptococcal infections of cows have been reported on a number of occasions. SANFELICE (12) isolated from a tumour in an ox a yeast which he described as being like *C. neoformans*, and MADSEN (6) found a strain of a species of *Cryptococcus* which seems likely

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to have been *C. neoformans*, in infected lymph nodes in a cow. POUNDEN, AMBERSON and JAEGER (11), and SIMON, NICHOLS and MORSE (13) described cryptococcal infections in herds in which mastitis was either one of the main symptoms or else was present in association with other manifestations.

As so little is known of the etiology of human cryptococcosis, and as there is a growing awareness that the disease is not a particularly rare one, the above observations are of considerable interest. In the past eight years seven cases of human cryptococcosis have been diagnosed in this country, five being of the meningeal type of the disease, and two being superficial lesions of the hand and ankle respectively. EMMONS (2) found *C. neoformans* in soils in North America, but a search for the organism in that substrate in New Zealand has so far been unsuccessful (DI MENNA, 9). The present paper records an examination of yeasts isolated from pooled raw milk.

MATERIAL AND METHODS.

Pooled raw milk was obtained from the Dunedin Milk Treatment Station at regular weekly intervals. Cultures were made on plates of Sabouraud agar which had been brought to a pH of 3–4 by the addition of 1 N HCl to the molten medium immediately before pouring. Culturing was used rather than animal inoculation, because there is evidence that more than one or two cells of *C. neoformans*, which theoretically at least would be detectable by this method, are necessary to initiate an animal infection.

Two series of cultures were made. The first was incubated at 37°C. thereby suppressing the growth of many of the non-pathogenic yeasts, and the second, which included a smaller number of samples and was for control purposes, was incubated at room temperature (c. 18°C.).

At first the samples to be cultured at 37°C. were centrifuged, the deposit taken up with a little saline, and spread on the plates. This procedure removed the bulk of the milk solids, which were undesirable because they partially neutralised the acid medium and so permitted heavier bacterial growth, and also because they produced an opaque fatty deposit which hindered examination of the plate cultures under a low power microscope objective. However comparative studies showed that not all the yeast cells had been deposited by centrifugation, but that many had been trapped by

the rising cream layer. For this reason the practise was discontinued early in the work and whole milk only was cultured.

The numbers of yeasts isolated per ml of milk varied so widely and erratically that it was not possible to identify or screen those recovered from an aliquot weekly portion. Instead, up to 6 ml of milk, 0.5 ml being spread on each 10 cm plate, were cultured and incubated at 37°C. each week. The colonies from as many plates as possible were subcultured into dextrose yeast extract broth for further examination. A proportion of the yeasts recovered were fully identified by the criteria of LODDER and KREGER-VAN RIJ (5), but the majority were merely screened for *Cryptococcus* species capable of growth at 37°C. Most of the isolates were fermenting yeasts, and in the screening process were eliminated by subcultivation into glucose yeast extract broth with Durham tube inserts. The non-fermenters were then inoculated upon the medium of MAGER and ASCHNER (7), upon which yeasts of the genus *Cryptococcus* synthesise a starch-like substance. Some non-fermenting yeasts of genera other than *Cryptococcus* are also capable of synthesising a starch upon this medium, but they are few in number and occurred only rarely among the strains which would grow at 37°C.

As the number of yeasts which would grow at room temperature was considerably greater than those which would grow at 37°C., the inocula used for these cultures were smaller, 0.15 ml of milk being spread on each 10 cm plate. Three plates were inoculated per sample and incubated in a dark cupboard for up to a week before examination. Usually only the colonies from the plate with the median count were subcultured, but when counts were low the yeasts from all three plates were treated. All strains recovered from the plates incubated at room temperature were identified.

In all 22 milk samples were examined, but room temperature cultures were made from only thirteen.

RESULTS.

Milk cultured at 37°C.

A total of 79 ml of uncentrifuged milk cultured at 37°C. yielded 1659 yeasts. No yeasts were recovered from three of the samples; the sample with the highest count yielded 88 yeast colonies per ml. Only 165 strains were recovered from the centrifuged deposit from 70 ml of milk. Of the yeasts isolated from both centrifuged and

uncentrifuged samples 265 were identified and a further 1314 screened for species of *Cryptococcus*. Two strains of *Cryptococcus albidus* (Saito) Skinner were recovered, both from the same sample of centrifuged milk. These strains were non-pathogenic to mice when injected intra-cerebrally, and could not be recovered from the brains of animals killed 2—3 weeks after inoculation. *Cryptococcus neoformans* was not found.

No lactose fermenting yeasts were found, and yeasts which could assimilate lactose made up only 10% of those identified. *Pichia fermentans* Lodder with its imperfect form *Candida krusei* (Cast.) Berkhout were together the commonest species, and the similar pair *Pichia membranifaciens* Hansen — *Candida mycoderma* (Rees) Lodder and Kreger-van Rij were next in order of frequency.

Milk cultured at room temperature (18°C.).

From 5.85 ml of milk cultured at room temperature 505 yeasts were isolated. Counts for individual samples varied between 0 and 297 yeasts per ml. Of the yeasts isolated in this section of the work 157 were identified. *Candida curvata* (Diddens and Lodder) Lodder and Kreger-van Rij, *Cryptococcus laurentii* (Kufferath) Skinner, and *Cryptococcus diffluens* (Zach) Lodder and Kreger-van Rij were the predominant species.

A detailed analysis of the species identified is given in Table 1.

DISCUSSION.

Although *Cryptococcus neoformans* was not isolated from the milks examined, the occurrence of strains of *Cryptococcus albidus* which would grow at 37°C. was interesting. Similar strains of *Cryptococcus laurentii* have been recovered from New Zealand soils (DI MENNA, 9), and the present finding again emphasises the necessity for complete identification of presumptive strains of *C. neoformans* by auxanograms and animal inoculation.

The absence of lactose fermenting yeasts was curious, for it is stated that these types are commonly present in both sweet and sour cream (HAMMER, 3). It is probable however that their initial concentration is very low, and that a preliminary enrichment technique is necessary to demonstrate them.

The sources of micro-organisms in milk are usually considered to be the cow, air, soil and utensils. PARLE (unpublished work)

TABLE 1.
Yeasts isolated from milk.

	Isolated at 37°C.	Isolated at room temperature
<i>Saccharomyces cerevisiae</i>	14	
<i>S. marxianus</i>	5	
<i>S. veronae</i>	1	
<i>Pichia membranifaciens</i>	6	
<i>P. fermentans</i>	22	
<i>Hansenula anomala</i>	1	
<i>Debaryomyces</i>		
<i>subglobosus</i>		4
<i>Cryptococcus laurentii</i>		22
<i>C. albidus</i>	2	2
<i>C. luteolus</i>		2
<i>C. diffluens</i>		22
<i>Torulopsis aerea</i>		1
<i>T. famata</i>	2	1
<i>Candida mycoderma</i>	57	3
<i>C. krusei</i>	100	15
<i>C. tropicalis</i>		1
<i>C. humicola</i>	1	1
<i>C. rugosa</i>	1	13
<i>C. macedonensis</i>	20	
<i>C. zeylanoides</i>	3	14
<i>C. utilis</i>	1	
<i>C. parapsilosis</i>	24	8
<i>C. robusta</i>	1	
<i>C. curvata</i>		27
<i>Trichosporon pullulans</i>		2
<i>T. cutaneum</i>	2	
<i>T. capitatum</i>		1
<i>Rhodotorula glutinis</i>		4
<i>R. mucilaginosa</i>	1	10
<i>R. minuta</i>		1
Unidentified	1	3
Total	265	157

has isolated *Hansenula anomala* (Hansen) H. and P. Sydow, *Trichosporon cutaneum* (de Beurm., Gougerot and Vaucher) Ota, and *Trichosporon capitatum* Lodder and Kreger-van Rij from cow faeces. Species of *Debaryomyces*, *Cryptococcus laurentii*, *Cryptococcus diffluens*, *Rhodotorula glutinis* (Fres.) Harrison and *Rhodotorula mucilaginosa* (Jorg.) Harrison were the commonest yeasts found in

air in Dunedin (DI MENNA, 8, 10). *Cryptococcus albidus*, *C. diffluens*, *Candida curvata* and *Trichosporon pullulans* (Lindner) Diddens and Lodder have all been found to be common in New Zealand soils (DI MENNA, 9 and unpublished work). Natural reservoirs of *Pichia membranifaciens*, *Pichia fermentans*, *Candida mycoderma* and *Candida krusei* have not yet been discovered in this country, but very little work has been done on this subject.

S u m m a r y.

1. Yeasts from 22 samples of pooled raw milk were either fully identified or else screened for the presence of *Cryptococcus neoformans*.

2. *C. neoformans* was not recovered, but two strains of *Cryptococcus albidus* which would grow at 37°C. were isolated.

3. No lactose fermenting yeasts were found, and the proportion of lactose utilisers among the isolates was not great.

4. It seems most likely that the majority of the yeasts recovered were contaminants of the milk from soil or air.

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STUDIES ON THE RADIATION INACTIVATION OF MICRO-ORGANISMS

IV. PHOTOREACTIVATION OF THE INTERMEDIATE STAGES IN THE TRANSFORMATION OF *BACILLUS* *CEREUS* SPORES INTO VEGETATIVE CELLS¹⁾

by

J. H. STUY

(Received July 23, 1956).

In an earlier study it was shown that the non-photoreactivability of the spores of two easily photoreactivable *Bacillus cereus* strains was not due to their lack of free water (STUY, 1956a). The writer suggested that this behaviour of the spores might be explained by their small metabolic activity. Accurate experiments have revealed that bacterial spores contain rather few active enzymes (SPENCER and POWELL 1952, POWELL 1955, LAWRENCE and HALVORSON 1954, STEWART and HALVORSON 1954, LAWRENCE 1955) and that they lack many of the ordinary ones (HARDWICK and FOSTER 1953). Assuming that photoreactivation (PHR) is not a direct restoration of UV-damaged sites in a cell, one may regard the enzymes as participating in the PHR-process are not present in the spores. This view is supported by the behaviour in this respect of viruses and spermatozoa. Viruses which are practically devoid of enzymatic activity, need the metabolic apparatus of the host cell for their PHR (DULBECCO 1949, BAWDEN and KLECZKOWSKI 1953). Spermatozoa exhibit an analogous behaviour in so far that PHR of UV-inactivated spermatozoa occurs only in the oocyte (BLUM, ROBINSON and LOOS 1951, MARSHAK 1949). They have a greater metabolic activity but this seems to be directed mainly towards fertilization.

¹⁾ Part 1, 2 and 3: Biochim. biophys. Acta **17**, 206; in press.

Since the vegetative cells of the *B. cereus* strains studied showed a great PHR, somewhere during the development of vegetative cells from the spores, the latter achieve the property of PHR. This paper is a report of the experiments which were undertaken in order to determine which stages in the transformation of the spores into bacteria can be reactivated by light after UV-inactivation. The results provide further evidence in favour of the above-mentioned view.

At the beginning of the experiments the problem was encountered that the transformation of the spores into bacteria was far from uniform. Some spores developed very rapidly, others very slowly into vegetative cells. But each spore underwent a series of changes in "colour" (phase-contrast microscopy) and in shape. It is possible to distinguish between five different stages of which the first one is the resting spore and the fifth the dividing bacterium. These stages had to be isolated. Two methods were used to perform this. The first method made use of improved growth conditions in order to make the transformation uniform. The disadvantage of this method was that no absolutely pure suspensions of the spores in the stages III and IV could be obtained. In the second method metabolic inhibitors were used. It was possible to inhibit completely the transformation of one stage into another. The disadvantage of this method is obvious, the cells obtained were physiologically damaged.

EXPERIMENTAL.

Micro-organisms, materials.

The p2 strain of *B. cereus* was used in all experiments.

Spore suspensions were obtained as described previously (STUY 1956a) using the method of CHURCH and co-workers (1954).

Suspensions of vegetative cells were prepared by growing the bacteria at 30°C. for 18 hours in brain heart infusion broth (BHI) under continuous magnetic stirring. The bacteria chains were broken up by means of a mild ultrasonic treatment. The cells were spun down and suspended in phosphate buffer pH 7.0.

After the operations the spores or bacteria were seeded in peptone agar (LAWRENCE 1955) and incubated at 30°C. for 18 to 24 hours.

The medium referred to below as AAT consisted of a solution of 40 μ moles/l adenosine, 2 mmoles/l l-alanine and 1 mmole/l l-tyrosine in 30 mmoles/l phosphate buffer pH 7.3.

The antibiotics were kept in the cold in stock solutions containing 1 mg per ml. They were prepared weekly.

All nutrient media were purchased from Difco.

UV-irradiation and illumination.

This was performed as described in a previous paper (STUY 1955). The UV-dose-rate was about $120 \mu\text{W}/\text{cm}^2$. In all experiments the Schott light filter WG3 (4 mm) was used. The distance between the lamp and the cuvettes was 15 cm.

RESULTS.

1. Transformation of resting spores into vegetative cells.

Stage I.

Very pure suspensions of resting spores were obtained by repeated centrifugings at 1000 r.p.m. Phase-contrast microscopy showed the spores to be ellipsoid-shaped "white" particles (Fig. 1)¹). Most of the spores had a "shadow" which was the membrane of the bacterium in which the spore had grown (Fig. 2). It was impossible to remove this membrane. The resting spores resisted a heating at 70°C. for 10 minutes. They were very UV- and X-ray resistant.

Stage II.

The resting spores were heated at 65°C. for 15 minutes. This heating stimulated subsequent "germination" (EVANS and CURRAN 1943). After the heating the spores were introduced into AAT and incubated at 37°C. They turned "blue" (PULVERTAFT and HAYNES 1951) and swelled to a certain extent but did not develop beyond this stage even after many hours of incubation (Fig. 3). According to POWELL and STRANGE (1953) the spores excrete calcium, dipicolinic acid and other nitrogen compounds. They take up water from the medium. Other changes which are caused by the AAT is the rapid loss of resistance against heat, UV- and X-rays (STUY 1956b).

Experiments showed that this first step of the transformation was not inhibited by large doses of UV-radiation or X-rays.

Stage III.

The stage II spores were spun down and re-suspended in BHI

¹) All photographs are of *B. cereus* p2. The magnification of the phase-contrast microscope photographs is about 3500.

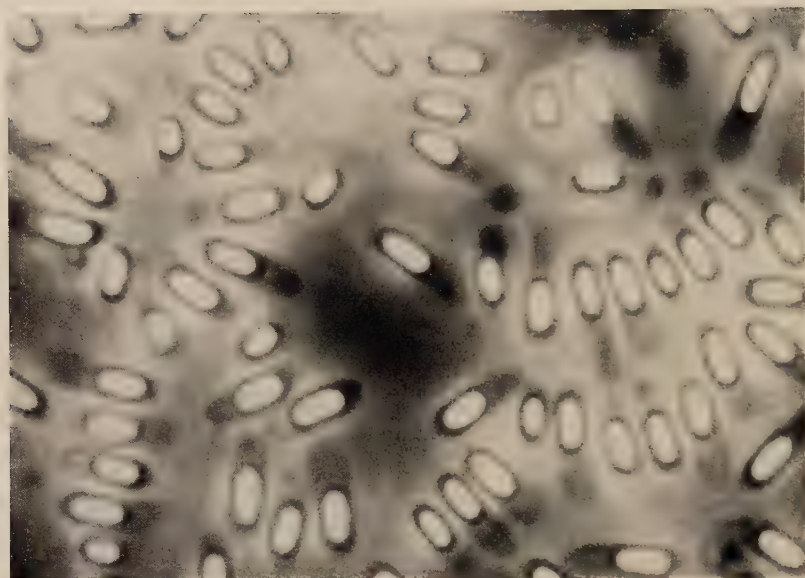


Fig. 1. Resting spores.



Fig. 2. Resting spores, electron microscope. Air-dried. Magnification 18000.

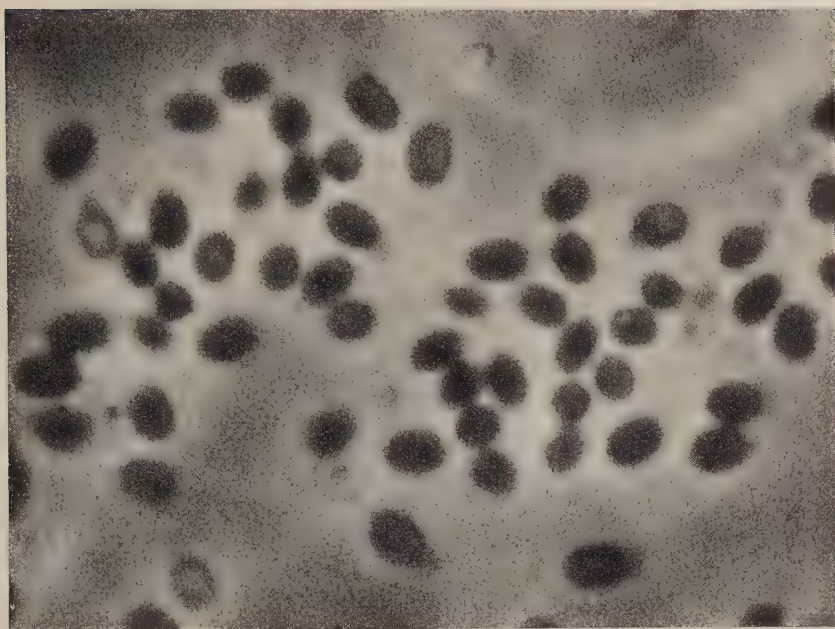


Fig. 3. Stage II spores.

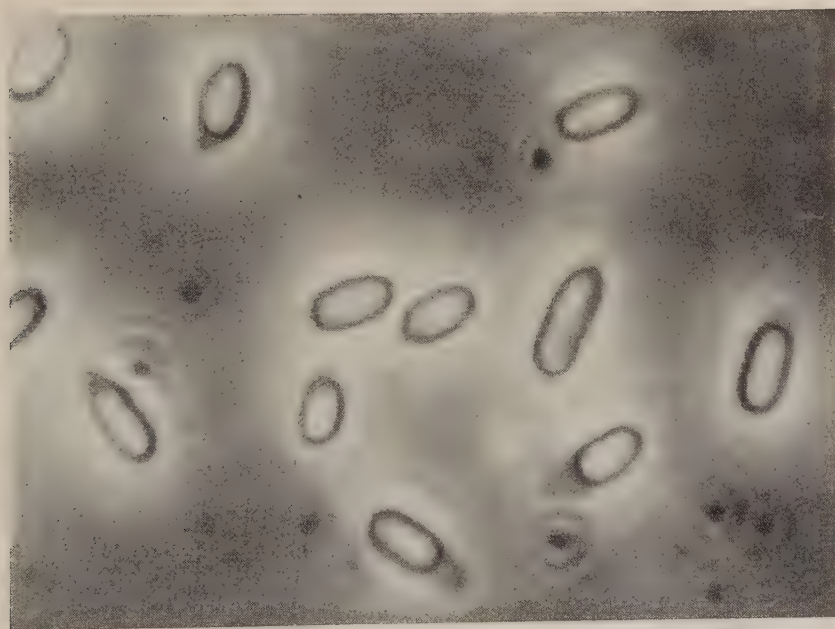


Fig. 4. Stage III cells.

with a pH of 7.5 to 7.6. After 30 to 50 minutes of incubation at 37°C. the spores had swelled considerably and turned less "blue" (Fig. 4). More than 90% of the cells were in this stage.

Less uniform transformation was obtained when the pH was decreased or with other nutrient media such as nutrient broth, yeast-extract (1%), peptone (1%) and hydrolyzed casein (1%).

By incubating the spores in BHI to which an inhibitor had been added, the transformation could be stopped at stage III. Aureomycin, terramycin, tetracyclin and chloroamphenicol (10 μ g per ml) were effective in this way. After 3 hours of incubation only stage III cells were observed. Half of them were still viable.

Fluoride (100 mmoles/l), cyanide (10 mmoles/l), sulfadiazin (100 μ g/ml) and penicillin (100 units/ml) were ineffective as inhibitor. Streptomycin killed the cells very rapidly.

Higher concentrations of the first-mentioned antibiotics inhibited the transformation into stage III.

Stage IV.

After 80 to 90 minutes of incubation of stage II spores in BHI pH 7.5 the suspension contained 50 to 80% cells which appeared



Fig. 5. Stage IV cells.

to be bacteria protruding out of the spore remnants (Figs. 5 and 6).

When, after 30 minutes of BHI-incubation, aureomycin, terramycin, tetracyclin or chloroamphenicol was added (10 $\mu\text{g/ml}$) the development stopped at stage IV. The cells were, however, longer than ordinary ones and often oddly shaped. Obviously the division mechanism was damaged. After 3 hours of incubation 50 to 90% of the cells had been killed by the antibiotics.

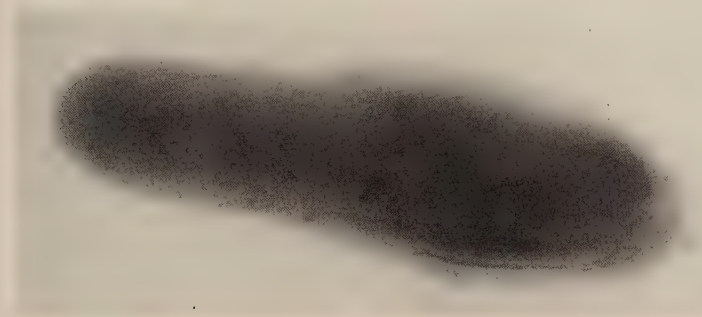


Fig. 6. Stage IV cell, electron microscope. Air-dried and non-shadowed. Magnification 24000.

Stage V.

Prolonged incubation of stage II spores in BHI resulted in intensive vegetative growth. These cells were designated as stage V cells.

General remarks.

It is of great importance that the spores be washed many times. Otherwise incubation in AAT may lead to a development beyond stage II. This was also observed when the spore concentration was too high. In general the experiments were performed with a cell concentration of 10^8 per ml.

A slight modification of the method by which the spores are prepared may result in a deterioration of the "germination" rate of the spores in AAT. This was observed twice.

The writer wishes to draw attention to the nomenclature of the transformation process and the intermediate stages. In his opinion there exists some confusion in this field. The term germination should be defined more clearly. The suggestion of MANDELS and coworkers (1956) is helpful but it does not go far enough. Since the intermediate stages are as yet nameless they are referred to in this paper as stage I or II spores and stage III or IV cells.

2. Photoreactivation of the intermediate stages.

Many experiments showed that stage III and stage IV cells could be reactivated by light after UV-inactivation. In table 1 some data are summarized. It was observed that stage III and stage IV cells which had been obtained by means of the inhibition method gave no PHR.

TABLE 1.

Photoreactivation of the stages of the transformation of *B. cereus* p2 spores into vegetative cells. UV-exposure time: for resting spores (stage I) 15 minutes, for the other forms 2 minutes. Illumination time: 60 minutes.

Stage	Number of viable cells per ml		
	initial	after UV	and after illumination
I	8.0×10^5	4.0×10^3	2.4×10^3
	5.5×10^5	8.5×10^2	4.7×10^2
	1.2×10^6	1.9×10^3	1.1×10^3
II	1.2×10^6	2.2×10^3	2.1×10^3
	9.0×10^5	4.9×10^3	3.5×10^3
	7.5×10^5	7.5×10^3	1.0×10^4
III	9.0×10^5	3.0×10^3	1.2×10^3
	7.5×10^5	8.5×10^2	8.0×10^4
	7.5×10^5	8.5×10^2	3.5×10^4
IV	7.5×10^5	5.5×10^1	1.7×10^4
	1.1×10^6	7.4×10^2	3.2×10^1
V	4.5×10^5	3.0×10^1	4.9×10^3
vegetative cells	1.3×10^6	1.2×10^2	3.7×10^4
	1.3×10^5	1.2×10^2	7.5×10^4

In order to find out how soon stage II spores showed PHR when they were incubated in BHI, the following experiment was carried out. Resting spores were preheated and incubated in AAT for 30 minutes. They were centrifuged down and resuspended in BHI which had already been warmed at 37°C. The cell concentration was about 10^8 per ml. After certain time intervals 0.1 ml samples were removed and diluted 100-fold with cold phosphate buffer pH 7.0. The number of viable cells was counted and the suspension was irradiated in the

cold. The UV-exposure time was 2 minutes. The irradiated suspension was illuminated also in the cold, giving maximum PHR and the "dark" and "light" survivors were counted. All counts were

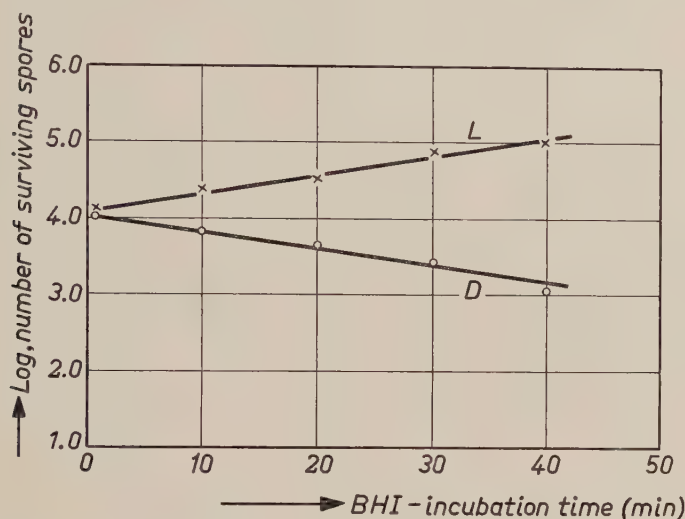


Fig. 7. Photoreactivation of stage II spores developing into stage III cells. Stage II spores were incubated in BHI. After certain times samples were removed, UV-irradiated for 2 minutes (curve D) and illuminated for 60 minutes (curve L).

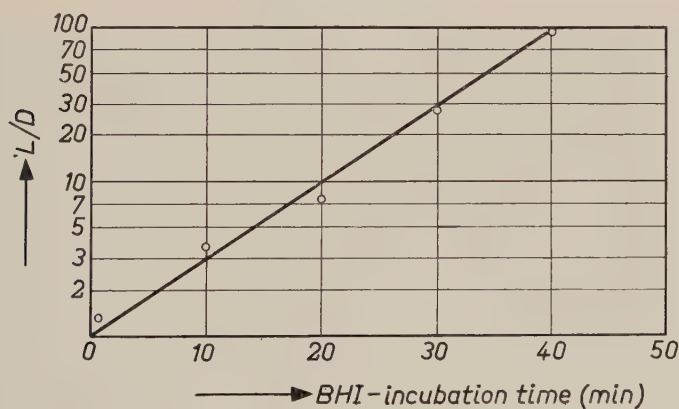


Fig. 8. Photoreactivation of stage II spores developing into stage III cells. Stage II spores were incubated in BHI. After certain times samples were removed, UV-irradiated for 2 minutes and illuminated for 60 minutes. The quotient of "light survivors" divided by the "dark survivors" is plotted against the incubation time.

carried out in quadruplicate. Figs. 7 and 8 show the results of such an experiment.

It was important to find out whether UV-inactivated stage II spores could be photoreactivated after incubation in BHI for a certain time. The answer on this question was negative, as shown by table 2.

TABLE 2.

Photoreactivation of UV-inactivated stage II spores after certain times of BHI-incubation. UV-inactivated stage II spores were suspended in BHI with a concentration of $10^3/\text{ml}$ and incubated at 37°C . At certain times 0.1 ml samples were removed, diluted 100-fold with cold phosphate buffer pH 7.0 and illuminated for 60 min.

BHI-incubation	treatment	number of surviving cells per ml
0	none	1.1×10^4
	60' light	1.1×10^4
30	none	7.5×10^3
	60' light	6.0×10^3
60	none	7.0×10^3
	60' light	6.0×10^3
70	none	7.0×10^3
	60' light	3.5×10^3

DISCUSSION.

Summarizing the results of the experiments described in this paper the following picture is obtained. Resting spores of the p2 strain of *B.cereus* show no PHR. During incubation in AAT, a synthetic medium permitting no vegetative growth, the spores develop into stage II spores. These are heat and UV-sensitive. They cannot be reactivated by light after UV-inactivation. After having introduced these spores into BHI, a rich and complex nutrient medium, they start growing, reach stage III and stage IV and finally they divide. Stage III and stage IV cells show PHR, in fact PHR is already observable after a few minutes of BHI-incubation.

In order to understand this phenomenon one requires a better insight in the metabolic activities of transforming spores. Much work has already been done by other investigators on the first step of this transformation but of the entire process little is known. From what has been published and from own experiments the writer has formed the following view. By introducing resting spores into

AAT a series of processes is initiated. These processes are all of a catabolic nature. They are not inhibited by common metabolic inhibitors or antibiotics, nor by UV or X-rays.

This indicates that some kind of "trigger" mechanism is involved. No synthesis of nucleic acids or proteins is observable. The metabolic apparatus of the spore remains small, although there may be an increase in the number of active enzymes due to some "unmasking" mechanism. This has not yet been reported. When the proper nutrients are supplied various syntheses start. An increase in DNA, RNA and catalase has been observed (FITZ-JAMES 1955, and unpublished experiments) and certainly all kinds of substances start being synthesized at a rapid rate. The metabolic apparatus of the spores is extended and in such a way that after some minutes of BHI-incubation the spores are already capable of PHR. Figs. 7 and 8 illustrate clearly that during BHI-incubation the photoreactivability of the spores increased. This was not due to the increase in UV-inactivation.

The experiments described here have been carried out with suspensions of growing cells. Caution is necessary in interpreting the results of such experiments in terms of one cell. Only if all cells transform uniformly such an interpretation is justified. Microscopical observations of the transforming stage II spores always revealed, that up to stage III there was a high degree of uniformity but thereafter some cells developed quicker than others. Since, at the beginning, the transformation was observed to be uniform, it seems justifiable to conclude that during the first 30 to 40 minutes of BHI-incubation of stage II spores, each spore increases its capacity to photoreactivate UV-damage. This might be caused by its expanding metabolic activity.

A c k n o w l e d g e m e n t.

The writer wishes to thank Professor K. C. WINKLER for his helpful advice and for many valuable discussions. He is grateful to Miss M. T. BORCHERT and Miss J. KRAMER who carried out the many experiments. The electron microscope photographs were made by Mr. H. HAANSTRA while Mr J. M. NIEUWENHUYZEN helped in taking the phasecontrast microscope photographs. To them the author's thanks are due.

S u m m a r y.

The transformation of *Bacillus cereus* spores into vegetative cells

has been studied. It was possible to distinguish between five different stages. Each stage could be isolated.

During the development of stage III cells from stage II spores the phenomenon of photoreactivation could be observed.

The results strongly suggest that the non-photoreactivability of UV-inactivated resting spores and stage II spores is due to their limited metabolic activity.

R é s u m é.

Le développement des cellules végétatives des spores de *Bacillus cereus* a été étudié. Il était possible de distinguer cinq états différents. Chaque état pouvait être isolé.

Le phénomène de la photoréactivation pouvait être observé pendant la transformation des spores de l'état II en de cellules de l'état III.

Les résultats indiquent nettement que l'absence de la photoréactivation des spores UV-inactivées de l'état I et de l'état II est due à leur activité métabolique limitée.

Z u s a m m e n f a s s u n g.

Die Entwicklung von vegetativen Zellen aus Sporen von *Bacillus cereus* wurde untersucht. Es war möglich um fünf verschiedene Stadien zu unterscheiden. Jedes Stadium konnte isoliert werden.

Während der Entwicklung von Stadium III Zellen aus Stadium II Sporen konnte die Photoreaktivierung beobachtet werden.

Die Resultaten deuten darauf, dass UV-inaktivierte Stadium I und Stadium II Sporen keine Photoreaktivierung zeigen weil ihre metabolische Aktivität zu beschränkt ist.

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A SIMPLE METHOD TO ABSORB NON- PRECIPITATING ANTIBODIES FROM IMMUNE SERUM

by

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Generally, antibodies can be removed from immune serum by incubating the serum with the corresponding antigen-containing microorganisms and removing the organisms by centrifugation. When the antigen is present in a purified state, no difficulties arise if its solution can be brought to precipitation with the serum in question.

In our work on the antibodies in syphilitic serum, we confirmed the finding of D'ALESSANDRO and DARDANONI (1953) in that this serum shows a positive complement fixation reaction with a protein antigen, isolated from *Treponema pallidum* (Reiter strain). We were unable to get a visible antigen-antibody complex by mixing this antigen with human syphilitic serum under several conditions.

In literature, methods for the isolation of antibodies are described, based upon the coupling of the corresponding antigens to insoluble carriers, followed by percolation of the sera. CAMPBELL, LUESCHER and LERMAN (1951) used diazotated p-aminobenzylcellulose while ISLIKER (1954) employed the acid chlorides of several synthetic resins.

As in our experiments we only needed the removal of antibody from serum, an absorption technique was developed which requires no chemical equipment and experience and which can easily be performed in the serological laboratory. It was observed in some cases that the Chamberland filter acts as a powerful adsorbent for antigen, while immune sera, even when filtered in relatively small quantities, showed no loss of titer. It was established that these facts also hold for the protein antigen-antibody system.

The first property is illustrated in table 1. A stock solution of protein antigen in saline (0.391 mg N/ml) was used. The sera were pools of human syphilitic sera. Antigen- and serum dilutions were prepared with veronal-buffered saline (pH 7.4). The complement fixation test (CFT) used was a highly standardized routine technique¹⁾.

TABLE 1.

Adsorption of protein antigen by passing a solution of this antigen through a Chamberland filter (L3).

a. CFT with syphilitic serum (1 : 5) before adsorption. The result shows the prozone phenomenon usually obtained with this antigen.

Antigen dilutions					
1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
—	—	+++	++++	++++	+

b. CFT with the same serum after adsorption.

Antigen dilutions					
1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
—	—	—	—	—	—

TABLE 2.

Selective absorption of antibody to protein antigen from syphilitic serum by passing a mixture of equal volumes of serum and antigen through a Chamberland filter (L3).

The optimal antigen dilutions employed in the CFT were: cardiolipin antigen 1 : 200 and protein antigen 1 : 80. The results are expressed as the highest serum dilutions giving positive reactions.

	Serum 1		Serum 2		Serum 3	
	CFT with card.ant.	CFT with prot.ant.	CFT with card.ant.	CFT with prot.ant.	CFT with card.ant.	CFT with prot.ant.
Serum + buffered saline	1 : 8	1 : 2				
Serum + antigen (1 : 10)	1 : 8	—				
Serum + antigen (1 : 40)	1 : 8	—				
Serum + antigen (1 : 160)	1 : 8	—				
Serum + antigen (1 : 640)	1 : 8	1 : 2				
Serum + buffered saline			1 : 64	1 : 8		
Serum + antigen (1 : 10)			1 : 64	1 : 2		
Serum + antigen (1 : 40)			1 : 64	1 : 4		
Serum + antigen (1 : 160)			1 : 64	1 : 8		
Serum + antigen (1 : 640)			1 : 64	1 : 8		
Serum, unfiltered					1 : 16	1 : 8
Serum + undiluted antigen					1 : 16	—

¹⁾ To be published.

In absorbing the antibody to protein antigen from syphilitic serum, antigen and serum were mixed just before filtering. As syphilitic serum also contains the classical cardiolipin antibody (reagin), the concentration of this antibody should not diminish on selective absorption. This is shown in table 2.

No attempts were made to dissociate the adsorbed antigen-antibody complex.

A c k n o w l e d g e m e n t s.

The author wishes to thank Dr L. DARDANONI (Palermo) for the generous supply of the Reiter strain of *Treponema pallidum*. He is indebted to Miss J. WERTENBROEK and Mr S. DE BRUIJN for technical assistance.

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DELAYED FERMENTATION OF SUCROSE BY CERTAIN HAPLOID SPECIES OF *SACCHAROMYCES*

by

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Among the procedures used for the identification of yeasts, the fermentation of certain sugars, judged by the formation of carbon dioxide, takes an important place. Whereas LODDER and KREGER-VAN RIJ (1952), following the earlier Dutch School started by STELLING-DEKKER (1931), have always made use of the Einhorn tube to demonstrate fermentation, in this laboratory the Durham tube method is preferred because it proved to be more sensitive and more convenient to handle (BOUTHILET *et al.*, 1949). According to the Dutch procedure final reading, as to the ability of a yeast to ferment a particular sugar, is made after 10 days. We have observed that certain yeasts belonging to the former sub-genus *Zygosaccharomyces* showed no fermentation of sucrose at room temperature during the first 20 to 25 days after inoculation. After this period, however, a distinct gas production began, often filling an inverted vial in about 2 or 3 days. The question arises as to the explanation and significance of this delayed fermentation and the possible taxonomic confusion it might create.

There are some indications in the literature that this phenomenon may have been responsible for certain contradictory results. SAITO (1907) described two supposedly non-sucrose fermenting yeasts, *Zygosaccharomyces japonicus* and *Z. soya*. Since SAITO used the Lindner slide technique to test for fermentation the incubation was of short duration and no gas formation was observed with sucrose.

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However, cells ground with quartz sand gave a distinct invertase test and the ground cells produced gas with sucrose in the Lindner slide test. Both species described by SAITO were subsequently tested by STELLING-DEKKER (1931), who found no fermentation of sucrose in Einhorn tubes in 2 weeks, but a slow non-delayed fermentation when the quantitative van Iterson-Kluyver fermentometer was used. In this instrument a heavy inoculum and anaerobic conditions are used. STELLING-DEKKER (1931) studied several other species of *Zygosaccharomyces* which fermented sucrose slowly in the quantitative fermentometer, but which had previously been described as non-fermenters of sucrose.

KARAMBOLOFF and KRUMBHOLZ (1931) described a new species, *Z. gracilis*, and stated that this yeast contained invertase, but did not ferment sucrose. HOFMANN (1934, 1936) demonstrated the presence of invertase in the supposedly sucrose negative BEIJERINCK strain of *Schizosaccharomyces octosporus* and isolated another strain of this yeast which showed a weak sucrose fermentation.

KLUYVER and CUSTERS (1939/1940) studied a number of glucose fermenting yeasts, including the BEIJERINCK strain of *Schiz. octosporus*, which clearly assimilated sucrose and certain other disaccharides, but did not ferment these under the usual testing conditions. However, by using the Warburg technique they found a weak fermentation of these disaccharides, although only in the presence of air.

HESTRIN and LINDEGREN (1950) studying a haploid, heterothallic strain of *S. cerevisiae* found that this yeast was inactive towards sucrose unless the cells were dried, frozen or aged. Although HESTRIN and LINDEGREN apparently did not test for a delayed fermentation of sucrose they appear to have been aware of such a possibility, as they stated, "The existence of latent enzyme systems suggests the further possibility that in some cases onset of fermentations after delay may be due to a non-adaptational alteration in the physiological state in the course of which enzyme systems originally latent in the cell are made operative". The role of cell permeability in sugar fermentation by yeasts has been investigated and reviewed by GOTTSCHALK (1946, 1949). The present study was undertaken in the hope of throwing more light on the mechanism and significance of the delayed fermentation of sucrose by certain yeasts.

The problem was approached by determining first whether or not the delayed fermentation involved mutation-selection, or

formation of inducible enzymes. In addition, the effects of certain physical treatments of the cells, variations in the medium and fermentation conditions, and the possible role of autolysis were considered in an effort to explain the phenomenon.

MATERIALS AND METHODS.

Cultures. The yeasts used and their origin are listed in Table 1.

Media. (a) The stock cultures were maintained on slants of 10°Brix unhopped malt agar, pH 5.5—5.8. (b) Fermentation tests were done in yeast autolysate (YA), 10 per cent v/v, containing 2 per cent of the desired sugar. The autolysate was prepared by the procedure of BOUTHILET *et al.* (1949).

Sterilization. The media were either autoclaved at 15 pounds pressure for 15 minutes, or sterilized by filtration through a Seitz asbestos filter, type ST, size L6 (Hercules Filter Corp).

Incubation. Unless specified otherwise the cultures were incubated at room temperature ($23 \pm 2^\circ\text{C}$).

Fermentation tubes. Durham tubes were used to determine gas production, i.e., test tubes 13×150 mm with inverted vials 9×30 mm, and 5 to 8 ml of medium. Quantitative measurements of fermentation were made in the van Iterson-Kluyver fermentometer (KLUYVER, 1914; KLEIN, 1933).

Sugar determinations. Reducing sugars were determined by HASSID's (1937) ferricyanide-ceric sulfate method. Sucrose was first hydrolyzed by a commercial aqueous invertase preparation following HASSID's procedure (1937).

Invertase. Activity of this enzyme was determined by the procedure of SUMNER and HOWELL (1935) discussed by NEUBERG and ROBERTS (1946). In most cases this method was modified by the use of M/15 phosphate buffer instead of acetate and 2 per cent instead of 6.5 per cent sucrose.

EXPERIMENTAL RESULTS.

Occurrence of delayed sucrose fermentation in species of *Saccharomyces*. Several species of the *Zygosaccharomyces* group, in which STELLING-DEKKER (1931) found a negative fermentation in Einhorn tubes, but a slow positive fermentation in the quantitative van Iterson-Kluyver fermentometer, were tested for the fermentation

of sucrose in Durham tubes. In addition, a strain of *Schizosaccharomyces octosporus* was included in view of the observations of HOFMANN (1934, 1936). A light inoculum was used taken from a 3 days old wort agar slant culture. Table 1 shows the days required for appearance of gas in the inverted vial. After gas formation started, the rate of CO₂ development from sucrose in the positive cultures was noticeably slower than that from glucose. Moreover, glucose is fermented without delay, which is also the case for maltose in several of the species listed. The fact that maltose can be fermented without delay indicated that the size of the molecule is not the limiting factor in fermentation. During the first 20—23 days of incubation, a gradual but distinct cell multiplication took place, which appeared to accelerate during the period of gas development. These experiments have been repeated numerous times with only a variation of 3 to 5 days before first appearance of gas. Since all cultures behaved more or less similarly further work was done only with the culture identified by us as *Z. gracilis* (#48—28). This yeast is now considered synonymous with *S. rouxii* (LODDER and KREGER-VAN RIJ, 1952). This strain was isolated from dried prunes.

Mutational and Adaptational Aspects. To explain the observed phenomenon, the possibility was considered that a sucrose fermenting variant might be produced spontaneously as was demonstrated for *S. chevalieri* in the case of galactose (WINGE and ROBERTS 1948, and SPIEGELMAN *et al.*, 1950). Such a variant would be expected to multiply and outgrow the non-fermenting cells, resulting in visible gas production.

S. rouxii was inoculated into 3 Durham tubes containing sucrose. All tubes showed gas after incubation for 25 days. Two days later, when the vials were about three-quarters filled with gas, the tubes were shaken to disperse the cells, and two loopfuls were transferred from each fermenting culture to 3 tubes each of the same medium. As controls, 2 tubes of glucose medium were similarly inoculated. The controls were actively fermenting in 24 hours, but the sucrose tubes only showed gas 32 days after inoculation. When the inoculum from a fermenting sucrose culture was increased to 0.1 ml, the time for the appearance of the first gas in a replicate test of 20 tubes varied between 21 and 25 days.

In addition, a loopful of each of the original fermenting sucrose tubes was streaked on 2 per cent sucrose — Y.A. agar. If mutant

TABLE 1.
Delayed fermentation of sucrose by various yeasts.

Species	Source	Amount of gas formed in inverted vials after specified no. of days at room temperature				
		20d.	23d.	28d.	30d.	32d.
<i>Zygosaccharomyces major</i> (<i>Saccharomyces rouxii</i>) ³⁾	CBS ¹⁾	—	—	2+	3+	3+ ⁴⁾
<i>Z. cavaræ</i> var. <i>beauverie</i> (<i>S. rouxii</i>)	„	—	1+	2+	3+	2+
<i>Z. japonicus</i> (<i>S. rouxii</i>)	„	—	—	—	1+	1+
<i>Z. japonicus</i> var. <i>soya</i> (<i>S. rouxii</i>)	„	—	—	2+	3+	3+
<i>Z. dairensis</i> (No. 2.13.1) (<i>S. rouxii</i> var. <i>polymorphus</i>)	„	—	1+	3+	3+	2+
<i>Z. barkeri</i> (<i>S. rouxii</i> var. <i>polymorphus</i>)	„	—	2+	3+	3+	2+
<i>Z. mellis-acidi</i> (<i>S. mellis</i>)	„	—	—	—	—	—
<i>Z. mongolicus</i> (<i>S. delbrueckii</i> var. <i>mongolicus</i>)	„	—	—	—	1+	1+
<i>Z. bisporus</i> (<i>S. bisporus</i>)	FT ²⁾	—	1+	3+	1+	1+
<i>Z. gracilis</i> (<i>S. rouxii</i>)	„	—	2+	2+	1+	1+
<i>Schizosacch. octosporus</i>	CBS	1+	2+	4+	2+	1+

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³⁾ Names in parentheses are recent synonyms or corrected diagnoses reported by LODDER and KREGER-VAN RIJ (1952).

⁴⁾ The indications 1+, 2+, 3+, and 4+ mean 1/4, 1/2, 3/4, and total Durham vial filled with gas.

cells were present, capable of utilizing sucrose, these cells would be expected to form larger colonies than the cells unable to attack sucrose. After 7 days at room temperature the colonies were still less than 1 mm in diameter, whereas on glucose — Y.A. agar they commonly reach 5 mm in diameter. Although growth on the sucrose plates was poor, 105 colonies were picked and inoculated into separate Durham tubes containing the sucrose medium. In all

cases it required between 23 and 28 days before gas formation started, whereas similar colonies inoculated into glucose, fermented it in 2 or 3 days. The above experiments are strongly indicative that mutation-selection is not operative in the ultimate fermentation of sucrose. Neither do the experiments support the idea that induction of sucrase is the main cause of the ultimate fermentation of sucrose.

Inversion of sucrose by dried, frozen and fresh cell preparations.

Since KARAMBOLOFF and KRUMBHOLZ (1931) demonstrated invertase in the type species of *Z.gracilis*, we tested our isolate for the presence of this enzyme. Since it is known that cell wall permeability is greatly affected by drying or freezing of the cells, invertase activity was compared in fresh, liquid-N₂ frozen and dried cells. The cells were grown for 3 days in 2 per cent glucose — Y.A. under shaking conditions in air and then centrifuged and washed several times in M/15 phosphate buffer at pH 5.2. The cells were resuspended in this buffer to make a concentration of 39 mg of dry weight per ml. Part of the suspension was centrifuged and dried for 24 hours at room temperature in vacuum over CaCl₂, by spreading the yeast cells on the wall of the centrifuge tube. The dried cells were then rehydrated in the same buffer solution. Another portion of the suspension was frozen in a Pyrex test tube by immersion for 10 minutes in liquid nitrogen, and stored at —20°C. until used. A third part of the suspension containing fresh, untreated cells was kept at 0°C. until used. To all 3 suspensions 0.03 M NaF was added to prevent fermentation during the inversion experiment. The flasks containing the suspensions were shaken to insure proper distribution of the cells and blanketed by N₂ to prevent respiration. Sucrose was added in a final concentration of 2 per cent. Samples were removed periodically and reducing sugars determined in the supernatant. In some cases, total sugar was determined after inversion by a commercial invertase preparation. The data are presented in Table 2. The figures show clearly that the freezing or drying treatment allowed an active inversion of the sucrose added, whereas even with the high concentration of cells used, the fresh cells showed very little activity. The frozen and thawed cells seemed to be slightly more active than the dried cells.

Since baker's yeast is known to secrete invertase very readily when the cells are damaged by freezing, drying or autolysis, it seemed

TABLE 2.

Inversion of sucrose by comparable amounts of dried, frozen, and fresh cells of *Saccharomyces rouxii* at pH 5.2.

Time, minutes	Reducing sugar as mg glucose per ml					
	Dried Cells		Liquid N ₂ Frozen Cells		Fresh Cells	
	total*		total*		total*	
	produced	present	produced	present	produced	present
0	2.10	19.80	2.60	20.60	2.00	19.80
60	6.70		7.75		2.10	
120	11.90		17.00		2.40	
150	15.00	19.50	19.30	19.80	2.30	19.30
180	16.40				2.54	

* Total after inversion with commercial invertase.

of interest to determine whether the observed delayed fermentation in *S. rouxii* might be due to a secretion of invertase into the medium due to aging of the inoculum. In order to test this the contents of 30 Durham tubes, all showing positive sucrose fermentation, were pooled and centrifuged. The supernatant was concentrated three-fold in vacuum and made to 30 ml by the addition of water and NaF solution to give a final concentration of 0.03 M NaF (pH 5.2). The cells were resuspended in M/15 phosphate buffer (pH 5.2) containing 0.03 M NaF and the volume was also made to 30 ml. Two per cent sucrose was then added and samples were withdrawn to determine reducing sugars. The cell suspension was shaken and kept under nitrogen as described before. The results are given in Table 3. In spite of the three-fold concentration, the supernatant culture liquid did not exhibit any invertase activity, whereas the aged cells, in contrast to fresh cells, were very active. These results show clearly that some time after inoculation the cell characteristics change, so that the invertase contained in the cells becomes operative, whereas, it is inactive in fresh cells (see Table 2).

This experiment was expanded by determining whether artificially induced autolysis by toluene might release the hydrolase from the cells as is the case with baker's yeast. *S. rouxii* was grown in glucose — Y.A. as described before, the cells were washed several times by centrifugation and a thick slurry made up (1 : 1 centrifuged cells and water). The mixture was allowed to autolyse at 37°C. for 3 days in the presence of toluene. The autolysate, which had a

TABLE 3.

Comparative inversion of sucrose by cells and supernatant of 25 day old cultures of *S. rouxii* which had shown delayed fermentation of this sugar.

Time in minutes	Reducing sugar as mg glucose per ml	
	Supernatant	Cells
0*	2.47	1.82
120	2.47	6.18
180	2.47	9.10
240	2.49	12.00
300	2.47	14.70
360	2.47	17.40
420	2.49	19.10
480	2.49	18.90

* Sucrose was added just prior to removal of zero time sample.

deep straw color and a pH of 5.2, was separated from the cell debris by centrifugation. The cell debris was washed with water and finally suspended in M/15 phosphate buffer pH 5.1 giving a heavy suspension. Both batches were made to the same volume, and NaF and sucrose were added to a concentration of 0.03 M and 1 per cent, respectively. Invertase activity was then determined as described before. The data are presented in Figure 1. Again the

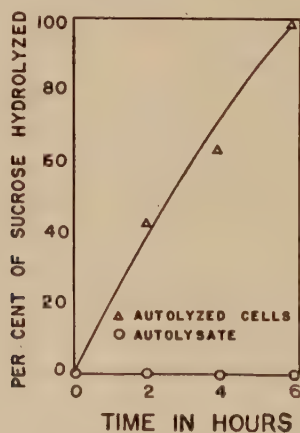


Fig. 1. Inversion of sucrose by toluene-autolyzed cells of *S. rouxii*.

results show a firm binding of the invertase to the cell debris in contrast to the release of the enzyme by similar autolysis of *S. cerevisiae* (NEUBERG and ROBERTS, 1946). It is noteworthy that

HOFMANN (1936) also found a retention of invertase by the cell debris of *Schiz. octosporus* autolyzed at neutral reaction.

Effect of pH on the rate of inversion of sucrose by dried cells.

It seemed of interest to characterize the invertase in *S. rouxii* by studying its activity at several pH values. According to HESTRIN (1949) betafructosido-invertase has its optimum at pH 4.5, and retains only 25 per cent of its maximum activity at pH 6.8. Alpha-glucosido-invertase, on the other hand, has maximum activity at pH 6.0, and retains 90 per cent of it at pH 6.8. The invertase activity of dried glucose grown cells was determined as described before, at pH 4.54, 5.62 and 6.85. The results are plotted in

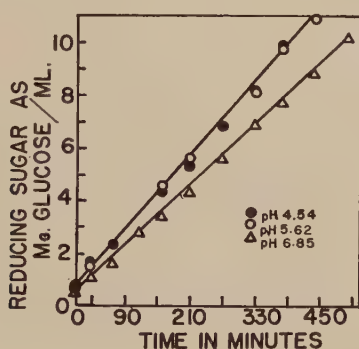


Fig. 2. Inversion of sucrose by dried cells of *S. rouxii* in phosphate buffers at different pH levels.

Figure 2. The lines show approximately equal rates of hydrolysis at the two lower pH values. Inversion was slower at pH 6.85 but not appreciably. Thus, based on the optimum pH values given in the literature, it would appear that *S. rouxii* contains both of the two known invertases. This belief is strengthened by the fact that dried cells were found capable of fermenting the fructose portion of raffinose, which is only possible when β -fructosidase is present.

Fermentation of dried versus fresh cells. Since it was shown that the invertase of *S. rouxii* becomes operative after careful drying of the cells, it was decided to compare the fermentation of glucose and sucrose by equivalent quantities of fresh and dried cells. The fermentations were carried out in the quantitative van Iterson-Kluyver fermentometer. The cells were grown for 3 days in 2 per cent glu-

cose-YA at room temperature under shaking conditions. After centrifugation and washing the crop with sterile water under aseptic conditions, an aliquot was dried rapidly (overnight) as described before and the dried cells were resuspended in the same original volume of sterile water. Four fermentometers were set up as follows:

Fermento- meter	Substrate	Cells	Final Substrate Conc.
1	0.7 ml 8% glucose – 20% Y.A.	0.7 ml fresh cell suspension	4% glucose – 10% Y.A.
2	0.7 ml 8% glucose – 20% Y.A.	0.7 ml dried cell suspension	4% glucose – 10% Y.A.
3	0.7 ml 8% sucrose – 20% Y.A.	0.7 ml fresh cell suspension	4% sucrose – 10% Y.A.
4	0.7 ml 8% sucrose – 20% Y.A.	0.7 ml dried cell suspension	4% sucrose – 10% Y.A.

The formation of gas was followed at room temperature and the data are presented graphically in Figure 3. Carbon dioxide volumes are not corrected for dissolved gas or calibration of the 1 ml ferment-

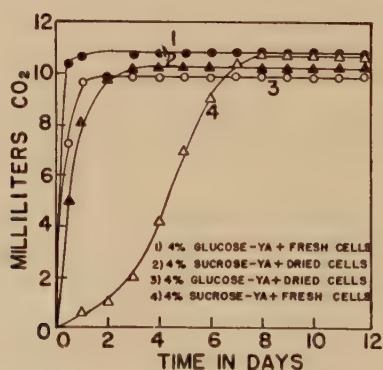


Fig. 3. Comparative CO_2 production from glucose and sucrose by fresh and dried cells of *S. rouxii* in the van Iterson-Kluyver fermentometer.

tometer volume. The curves show that the ability to ferment glucose of the dried and fresh cells was nearly the same. It can also be seen that after a short lag period the fermentation of sucrose by the dried cells was practically as rapid as the glucose fermentation by dried cells, whereas, the fermentation of sucrose by fresh cells was slow and required 8 days for completion. In contrast to the Durham tubes,

the induction period for the start of gas development was quite short in this case. There are two differences in experimental conditions between the fermentometer and the Durham tubes. In the former, the inoculum is much greater, and secondly, the conditions are strictly anaerobic. The effect of these two factors will be considered below.

This fermentation experiment was repeated with liquid - N_2 frozen cells and essentially the same results were obtained.

These results strongly suggest that permeability difficulties are involved in the delayed fermentation of sucrose by certain yeasts.

Effect of added glucose. This test was intended to show whether or not the availability of a source of ready energy, glucose in this case, would increase the rate of penetration and induce a more rapid fermentation of sucrose. Underlying this is the thought, that some form of energy is likely to be required for passage of sucrose across the cell membrane. The stimulating effect of small amounts of glucose on the fermentation of maltose has been shown by SCHULTZ and ATKIN (1939). The following van Iterson-Kluyver fermentometers were set up:

1. 2.5% glucose plus 3.33% sucrose in Y.A.
2. 2.5% glucose in Y.A.
3. 3.33% sucrose in Y.A.

Gas production was followed at room temperature and is plotted in Figure 4. The curves show that the fermentation of glucose in (1) and (2) was almost complete in 24 hours. In the case of the

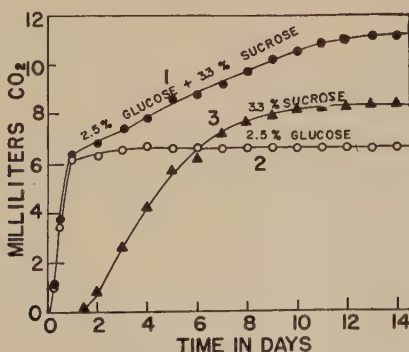


Fig. 4. The effect of the presence of glucose on fermentation of sucrose by *S. rouxii* in the van Iterson-Kluyver fermentometer.

mixture (1) a sharp break occurs in the curve when the glucose has been fermented completely, since curves (1) and (2) coincide until this point. Curve (3) and the second section of curve (1) representing the fermentation of sucrose differ appreciably in slope and total gas produced. The rate of sucrose fermentation in the mixture was much slower and less complete than when sucrose alone was used. The reason for the weaker fermentative power in this case is not clear.

Size of inoculum. It has been pointed out that the onset of sucrose fermentation in the van Iterson-Kluyver fermentometer comes much sooner than in Durham tubes. The possibility that the large inoculum used in the former might be responsible for this, was considered. To check this, variation in the size of the inoculum was studied using Durham tubes. The experiments clearly demonstrated that a heavy inoculum shortened the time for first appearance of gas and the total fermentation time. Maximum gas production with the heaviest inoculum used was reached in 8 to 9 days. This time is comparable to the period for maximum gas production from sucrose in the quantitative fermentometer, but is appreciably longer than that exhibited by dried or frozen cells.

Effect of anaerobiosis. Next, the effect of anaerobic conditions in Durham tubes was studied using the customary light inocula. An experiment was set up in which glucose and sucrose were tested under aerobic and anaerobic conditions. Freshly autoclaved and rapidly cooled media were used. Anaerobic conditions were obtained by flushing with N_2 and insertion above the regular plug of another plug soaked in alkaline pyrogallol, followed by a rubber stopper. All tubes were incubated at room temperature and the results appear in Table 4.

The data of Table 4 show that under strictly anaerobic conditions, using light inocula, neither glucose nor sucrose was fermented. It is apparent that whenever air was given access to the medium, fermentation of either sugar took place. It is of interest that tube (i) showed gas more rapidly after air was admitted, than did tube (h). The only difference between (h) and (i) was that the cells were older in tube (i) at the time air was admitted. The effect of age of the cells is discussed below.

It has been known since the time of PASTEUR that yeast requires

TABLE 4.

The effect of anaerobiosis on the production of gas from 2% glucose-YA and 2% sucrose-YA in Durham tubes

Days	Aerobic					Anaerobic				
	Glucose		Sucrose			Glucose		Sucrose		
	a	b	c	d	e	f	g	h	i	j
1	2+	2+	—	—	—	—	—	—	—	—
2	4+	4+	—	—	—	—	—	—	—	—
15			—	—	—	—	—	—	—	—
16			—	—	—	(*)	(*)	(*)	—	—
17			—	—	—	(+)	(+)	(—)	—	—
18			—	—	—	(1+)	(2+)	(—)	—	—
19			—	—	—	(3+)	(3+)	(—)	—	—
20			—	—	—	(4+)	(4+)	(—)	—	—
21			+	+	+			(—)	—	—
22			2+	1+	1+			(2+)	—	—
23			3+	3+	2+			(3+)	—	—
24			4+	4+	4+			(4+)	—	—
40									(*)	—
41									(1+)	—
42									(3+)	—
43									(4+)	—

(*) Tubes exposed to air by removal of cotton plug containing alkaline pyrogallol.

minute quantities of oxygen in order to grow when inoculated lightly into an otherwise suitable medium. Hence, lack of growth under the strictly anaerobic conditions is probably responsible, at least in part, for the absence of fermentation. More recently SCHULTZ *et al.* (1940) showed that oxygen, under appropriate conditions, shortens appreciably the induction period for maltose fermentation by baker's yeast. These workers believe that oxygen affects the permeability of the cells to maltose. Similar conclusions were drawn by KLUYVER and CUSTERS (1939/1940) for the fermentation of disaccharides by certain other yeasts.

Effect of the age on the inoculum. Several indications were obtained during the preceding experiments that old cells required less time to start the fermentation of sucrose than young cells. HESTRIN and LINDEGREN (1950) also noted that their haploid breeding stock fermented sucrose more rapidly when the cells were aged. A syste-

matic study was, therefore, made on the effect of age of the inoculum. *S. rouxii* was inoculated evenly over the entire surface of 100 ml 10 per cent malt agar, which had been allowed to solidify in a one-pint Blake bottle lying in a horizontal position. The yeast was allowed to grow at room temperature and periodically samples of the growth were removed from the center of the agar surface and inoculated in triplicate into Durham tubes containing sucrose medium. The inocula were light and roughly the same in all cases and were made directly with a needle. Table 5 shows how the time for appearance of gas varied with the age of the inoculum. The tabulated results show quite strikingly that the age of the inoculum has a profound effect on the time required for visible gas production. It is noteworthy that the total age of the cells, i.e., the period on the agar surface plus the average period in Durham tubes prior to the beginning of fermentation, was approximately constant (about 28 to 30 days). It was shown above that in cells of this age the invertase had become operative. It would, therefore, seem that after sufficient aging the cells begin to invert the sucrose and this in turn would allow an active fermentation and an increase in growth rate at the expense of the invert sugar formed.

Table 5.

Effect of age of culture prior to inoculation into 2% sucrose-YA on time required for appearance of gas

Age of inoculum, days	Time for appearance of gas*, Days			Total age of cells in days at time of the start of fermentation
	Tube			
	A	B	C	
6	22	22	23	28
8	20	19	20	28
11	19	18	18	29
14	17	15	16	30
18	12	15	13	31
24	2	2	2	26
25	2	2	2	27
28	2	3	2	30

* Appearance of gas means 1/4 vial or more of gas.

Effect of initial pH level on time required for appearance of gas.

ØRSKOV (1945) pointed out that the permeability of baker's

yeast to various substrates was greatly affected by the pH of the suspending medium. In order to test whether the initial pH of the medium had any effect on the time required for the onset of fermentation, the pH of the usual sucrose-YA fermentation medium was varied between 3.0 and 7.0 with increments of about 0.5 unit. The pH was adjusted by addition of HCl or NaOH and all media were sterilized by Seitz filtration. Durham fermentation tubes were used and glucose was used as a control. All the glucose tubes fermented in 24 to 48 hours, but in the sucrose series gas formation was delayed for 28 days and occurred only at pH 5.3 (natural pH of the sucrose-YA) and at pH 4.6. It was, therefore, concluded that variation of pH did not increase the permeability of sucrose in the case of *S. rouxii*.

Effect of sucrose concentration.

Since many species belonging to the sub-genus *Zygosaccharomyces* are remarkably osmoduric and the species under investigation was isolated from dried prunes having a sugar content of about 60 per cent, the effect of sucrose concentration on the delayed fermentation was investigated next.

Solutions of sucrose of 2, 5, 10, 35 and 50 per cent by weight in YA were used. The solutions were sterilized by Seitz filtration and dispensed aseptically into sterile Durham fermentation tubes (5 ml/tube). The entrapped air was removed from the inverted vials by evacuation. The experiment was done in quintuplicate and the tubes were inoculated with 0.05 ml each of a dense water suspension of 3 days old cells grown on wort agar and the tubes were stored at room temperature. It was found that at the 50 per cent sucrose level gas first appeared after 14 days, whereas, it took 24 days at the 2 per cent concentration. At the other sucrose levels, the times for first appearance of gas were intermediary between 14 and 24 days. The replicate determinations did not vary by more than one day.

It is apparent that a delayed fermentation still occurs even at the high concentrations. If increasing the concentration of sucrose made an increased number of sucrose molecules available with the minimum kinetic energy for active or passive transport across the cell membrane, a slow but not a delayed and abrupt fermentation might be expected. It is possible that the higher concentrations of sucrose helped to reduce the life of the viable cells, or changed membrane characteristics more rapidly.

DISCUSSION.

In the introduction we have alluded to the possible taxonomic confusion which might result from the fermentation of sucrose delayed for various periods depending on the previous history of the cells and on the method to test the fermentation. The present study has shown clearly that *Schiz. octosporus* and certain species of *Zygosaccharomyces*, several of which are now considered synonymous with *S. rouxii*, contain the necessary enzymes to ferment sucrose as rapidly as glucose. Yet, if young cells are lightly inoculated into Durham tubes or comparable fermentation tubes, sucrose fermentation does not become evident until 3 to 4 weeks after inoculation. Even though the fermentation of sugars is considered an important criterion for species differentiation, it would seem to us that, for practical reasons, it is preferable to disregard for taxonomic purposes the potential ability to ferment such sugars after a long delay. Since the delayed fermentation appears to be due to a change in the permeability of the cell walls because of aging and not to the formation of inducible enzymes or to mutation and selection, it would seem justifiable to disregard the latent sucrase. The experiments have reemphasized the necessity of standardizing the fermentation test, by using a moderate to light inoculum of young cells. Older cells, depending on their age, show a proportionately shorter delay with respect to the onset of fermentation.

The locus of the invertase in *S. rouxii* may be different from that in *S. cerevisiae* in which it has been considered to lie near the surface of the cells and where it can hydrolyze sucrose at a rate which greatly exceeds the fermentative capacity (WILKES and PALMER, 1932; NELSON and WILKES, 1932; DEMIS *et al.*, 1954 and ROTHSTEIN, 1954). A further difference is evident from the fact that at least the bulk of the invertase of *S. cerevisiae* goes readily into solution following toluene treatment or autolysis of the cells, whereas cells of *S. rouxii* retain their sucrase after autolysis, drying, freezing in liquid nitrogen and after aging. HOFMANN (1936) also observed retention of invertase by lysed cells of *Schiz. octosporus*. It is of interest to note that DAVIES (1955) found glucose grown cells of *S. fragilis* incapable of fermenting or hydrolyzing lactose, but after treatment of these cells with cetyltrimethylammonium bromide fermentation of lactose took place.

The experimental evidence suggests that first the inoculum shows a

slight growth, probably at the expense of the yeast autolysate. The cells then go through an aging process of at least 3 weeks, after which fairly suddenly the invertase contained in the cells becomes operative and hydrolyses the sucrose. This results in fermentation of the invert sugar and produces some additional growth. It appears immaterial whether the cells age in the fermentation medium or on a wort agar medium.

S u m m a r y.

Several haploid species of *Saccharomyces* and *Schiz. octosporus* were shown to ferment sucrose in Durham tubes after a delay of 3 to 4 weeks. Detailed studies were done with a strain of *S. rouxii*. The delayed fermentation of sucrose was not caused by mutation-selection or by inducible enzyme formation, since young glucose grown cells after drying, freezing, aging or autolysis contained an active sucrase. Cells pretreated by drying or freezing fermented sucrose nearly as fast as glucose. After autolysis, the sucrase of *S. rouxii* is only present in the cell debris and not in the autolysate. The use of a heavy inoculum in the van Iterson-Kluyver fermentometer resulted in a slow, but non-delayed fermentation. Variation in the pH or sucrose concentration had little effect on the delayed fermentation. It is suggested that after sufficient aging of the cells, the cell wall permeability undergoes a rather abrupt change, allowing the sucrose to come in contact with the sucrase of the cells.

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MICROORGANISMS OF THE *PLEUROPNEUMONIA* GROUP (FAMILY OF *MYCOPLASMATACEAE*) IN MAN

I. CULTURAL AND BIOCHEMICAL CHARACTERISTICS

by

A. G. M. HUIJSMANS-EVERS and A. CHARLOTTE RUYSS

(Received May 24, 1956).

Members of the *Pleuropneumonia* group of microorganisms (P.P.L.O.) cause well defined illnesses in animals for example pleuropneumonia in bovines, agalactia in goats, arthritis in rats. The finding of organisms of this group on the mucous membranes of man raised the question whether these too were pathogens or not. Strains were isolated from the urogenital tract of males and females with and without symptoms of inflammation and from the buccal mucosa of healthy people.

A great difficulty in the study of P.P.L.O. has been the resemblance between these organisms and the so-called L form of bacteria which is a phase in a growth cycle of various bacteria. Bacteriologists are divided in two groups. One considers the P.P.L.O. to be stabilized L variants of bacteria. The other group of bacteriologists stresses the differences in morphology, in metabolism and growth requirements between P.P.L.O. and L form.

The main characteristics of these organisms are the absence of a compact cell wall and the extreme softness of the cytoplasm. Spherical forms, minute corpuscles and sometimes filaments can be recognised. Cultures grow in fluid and on solid media. On the latter the colony grows down into the medium. Cholesterol is an essential nutrient for most of the P.P.L.O. strains. All are resistant to penicillin.

A provisional classification scheme has recently been proposed by EDWARD and FREUNDT (1956) who define a new order different

from the *Schizomycetes*, to which belongs the Family of the *Mycoplasmataceae* with the genus *Mycoplasma*. This genus is subdivided into 15 different species. The type species is the organism of bovine pleuropneumonia named *Mycoplasma mycoides*. Four have been found in man, namely *Mycoplasma hominis* type 1 and 2, *Mycoplasma fermentans* (human type 3) and *Mycoplasma salivarius* (human type 4). Differentiation has been effected by biochemical and serological methods.

The aim of this study was to isolate a number of human strains from various sources and to try to differentiate these strains in order to investigate the possibilities of a correlation between the condition of the patient and the specificity of the strain.

METHODS.

Media. We used a heart infusion-peptone-broth which is boiled with 7½% of sheep- or goat blood and then filtered. To this broth 30% of horse serum is added and 2% agar for solid media (medium of KLIENEGER, slightly modified). To make the medium more selective thalliumacetate (1 : 8000) was added or two drops of penicillin (1000 U/ml) were spread over half of the surface of a solid medium shortly before use. All cultures were incubated both aerobically and anaerobically, the latter in a McIntosh jar at 37°C. Subcultures in fluid media were made by putting a block of agar with the growth into the broth. On solid media a block of agar was moved, with colonies facing downwards, over the surface of the new medium. A search for growth on solid media was made with 10 or 40 times magnification after 3 to 10 days incubation. Growth in fluid media was not always visible and was checked in each case by subculture on solid media. All strains were stored freeze-dried in the fluid medium.

Microscopy. Morphology was studied with phase contrast microscopy of colonies in situ on thin slices of the medium. DIENES' method of vital staining with methylene blue was also used. In several cases we tried the agar fixation technique of KLIENEGER-NOBEL (1950). In the typical colonies ordinary bacterial forms were invariably absent, in smears only irregular structures were observed. The colonies showed some differences in their surface structure, some formed a film and spots between the colonies (see below). Fermentation of sugars was tested on solid media with 30%

ascitic fluid, thallium acetate 1 : 8000 and with 1% of the various carbohydrates (glucose, levulose, saccharose, maltose, galactose, mannitol, mannose, dextrine and glycogen). The indicator was phenol red 0.005%.

Hemolysis was tested on horseblood-agar consisting of two layers, the bottom one being made of the ordinary P.P.L.O. medium with thallium acetate. This was covered by a thin upper layer of the medium with 5% horse blood. Hemolysis was judged always after 10 days' incubation of the strain because with some it became apparent only after 7—10 days.

M a t e r i a l. Examinations were carried out in male patients suffering from non-specific urethritis, in female patients with gynaecological complaints, in healthy women in the first half of pregnancy and in a group of women 6 weeks after delivery. A small number of miscellaneous specimens of other origin was examined too.

In all patients the bacterial flora was examined in smears stained by Gram's method in order to obtain an impression of the degree of inflammation, if present, and to exclude any gonorrhoeal infection. In addition, a search was made for *Trichomonas vaginalis* in wet slides from all women and a few men.

In 27 healthy students the buccal mucosa was examined for the presence of P.P.L.O. For more details the reader is referred to the thesis of one of the present authors (HUIJSMANS-EVERS, 1955).

RESULTS.

I s o l a t i o n o f s t r a i n s. Table 1 summarizes the results of the examination of 125 persons, 56 of whom were patients. From the two male patients in whom gonococci were found no P.P.L.O. were cultivated. There were two male patients who carried *Trichomonas vaginalis*, one of them was positive for P.P.L.O. too, the other was not. Three patients with P.P.L.O. in their urethral discharge complained of pain in their joints. However, no culture from the synovial fluid could be made.

In women the frequency of positive cultures of P.P.L.O. increased parallel with the replacement of Lactobacilli in the vaginal discharge by other bacteria (see Table 2).

I d e n t i f i c a t i o n o f P.P.L.O. s t r a i n s.

All oral strains and a few strains isolated from the genital tract

TABLE 1.

conditions	number	P.P.L.O.	Trich. vag.
1. ♂ non specific urethritis	11	4	
former venereal diseases			
♂ non specific urethritis	15	9	
no venereal diseases			
♂ gonorrhoeal urethritis	2	0	
♀ gynaecological disorders			
vaginal discharge +++	13	10	4
" " +	7	2	0
pus bartholinitis	3	0	0
" salpingitis	1	0	0
	52	25	4
2. ♀ first half of pregnancy	22	5	0
♀ 6 weeks after delivery	20	8	0
	42	13	0
3. miscellaneous			
pleural fluid	1	0	
synovial fluid	2	0	
iritis	1	0	
	4	0	
4. buccal mucosa	27	23	
healthy students			
total	125	61	

TABLE 2.

Frequency of P.P.L.O. and Lactobacilli in vaginal discharge.

conditions	Lactobacilli predominant		Lactobacilli scarce		Lactobacilli absent		Total	
	No.	PPLO pos.	No.	PPLO pos.	No.	PPLO pos.	No.	PPLO pos.
gynaec. disorder	5	1	6	3	9	8	20	12
1st half of pregnancy	14	1	5	1	3	3	22	5
6 weeks after delivery	2	0	6	3	12	5	20	8
	21	2	17	7	24	16	62	25

did not grow aerobically. The other ones grew well under both conditions. Two genital strains could be subcultured through many generations on media without serum or ascitic fluid. All the other strains needed the richer substrates. The colonies sometimes showed a foamlike structure, in other cases they had a more granular surface

with a more or less coarse aspect. Other ones were smooth. On horse serum media some strains formed a fanlike film and spots between the colonies after 10 days incubation. According to EDWARD these films consist of cholesterol and phospholipids and the spots of calcium and magnesium soaps. Those strains which fermented sugars strongly did so with glucose, levulose, sucrose and maltose. With the other ones tested acid formation was slight and often variable. Colony morphology and biochemical behaviour allowed of differentiation into various groups (Table 3) which to a large extent were in conformity with the new species named by EDWARD and FREUNDT (1956). The differences will be discussed later. For comparison two bovine pleuro-pneumonia strains were included in our series, and a G strain from RUITER and WENTHOLT (1953)¹).

TABLE 3.
Determination of P.P.L.O. strains.

Source of strains	No.	growth			colonies	film spots	visiblity fluid media	ferment sugars	hemo-lysis after 10 d.	classifica-tion
		aerob.	an-aerob.	media not enriched						
I uroge-nital tract ♂	7	+	+	—	from smooth to foam structure	—	—	—	—	<i>M. hominis</i>
	21	+	+	—		—	—	—	—	<i>M. hominis</i>
II uroge-nital tract ♂	1	—	+	—	fine granular	+	±	+	+	<i>M. fermentans</i>
	3	—	+	—		+	+	+	+	<i>M. fermentans</i>
III uroge-nital tract ♂ U7	1	+	+	+	granular coarse	—	—	—	—	?
	1	+	+	+		—	—	—	—	?
IV buccal mucosa	17	—	+	—	smooth	+	±	—	+	<i>M. salivarius</i>
V P.P. bovine strains	2	+	—	—	coarse granular	—	+	+	+	<i>M. mycoides</i>

As is shown in table 3 the 51 human strains could be subdivided into 4 groups. The largest part of the urogenital strains fell into a group which conformed to the description of *Mycoplasma hominis* (EDWARD and FREUNDT). Three of our vaginal strains proved to be identical in morphology and biochemical behaviour with RUITER

¹) We wish to express our thanks to Drs RUITER and WENTHOLT for supplying various strains for comparison and to Dr DUREL from Paris for sending us a bovine strain.

and WENTHOLT's G strain. According to NICOL and EDWARD the G strain does not give hemolysis after 4 days incubation.

In our experiments, however, it was shown that hemolysis did become apparent after 7—10 days incubation in the G strain as well as in the three of our vaginal strains which behaved identically. Other urogenital strains never showed hemolysis. We found all our oral strains hemolytic too, which is contrary to the findings of EDWARD. The classification given in Table 3 was confirmed by serological methods which will be described in a following paper in which also the role of these microorganisms in man will be discussed.

Summary.

Sixty one strains of pleuropneumonia-like organisms (P.P.L.O.) have been isolated in man: 25 from the discharge of patients with urogenital disorders, 13 from women who were pregnant or who had borne a child 6 weeks previously. In women the frequency of positive cultures increased with the disappearance of *Lactobacilli*. Twenty three strains were cultivated from the buccal mucosa of 27 healthy subjects. Classification by means of cultural appearance and biochemical behaviour allowed of differentiation into 4 groups, three of which were in conformity with three species of *Mycoplasma* described by EDWARD and FREUNDT. The only difference as compared with this latter classification is that with our method *M. fermentans* and *M. salivarius* regularly gave clearcut hemolysis, which was not found by EDWARD. Two urogenital strains which grew well on not specially enriched media could not be identified.

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MICROORGANISMS OF THE *PLEUROPNEUMONIA* GROUP (FAMILY OF *MYCOPLASMATACEAE*) IN MAN

II. SEROLOGICAL IDENTIFICATION AND DISCUSSION OF PATHOGENICITY

by

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In a previous paper (this journal page 371) we described a number of P.P.L.O. strains isolated in man and we gave a provisional classification based upon their morphological and biochemical behaviour. To complete these data the serological methods of identification are described in the following.

We used complement-fixation tests and growth inhibition by anti-sera (EDWARD and FITZGERALD, 1954). Agglutination reactions did not give satisfactory results because of too little stability of the suspensions of the microorganisms which generally were also agglutinated by the control sera. An attempt to use antigens adsorbed to erythrocytes failed.

METHODS.

The antigens for the immunization of rabbits were made from 35—40 ml of broth cultures of the various strains. After incubation for 3 days the broth was centrifuged for 20 minutes at 12000 r.p.m. The sediment was washed twice with saline and resuspended in 1 ml saline. Rabbits were immunized intravenously twice weekly during 4 weeks with $\frac{1}{2}$ ml of this suspension. To obtain a serum which showed growth inhibition immunization had often to be continued for another four weeks or even longer. Ten days after the last injection the rabbits were bled and the sera were stored at — 20°C without preservative.

Sera for the complement fixation were prepared with one bovine strain (A) of pleuropneumonia, with three oral P.P.L.O. strains (T, K4, K19), with two ordinary genital strains (V32, U2) and with one of the hemolytic vaginal strains (V26). The antigens for the complement fixation test (C.F.) were prepared in the same way as those used for the immunization of the rabbits. If they proved to be anticomplementary they had to be washed a third time. Generally the suspensions showed a homogeneous turbidity. The sediment of the same amount of non inoculated broth was used as a control antigen. This was also slightly turbid.

The dosage of complement used in the main test was $1\frac{3}{4}$ times that found to give complete lysis in the preliminary titrations of the hemolytic system. The sera were inactivated in a $1/4$ dilution for 20 minutes at 56°C .

All tests were performed with a micromethod used in this laboratory with standard drops put into holes of plastic plates. These were incubated during one hour at 37°C .; then the hemolytic system was added and incubation was prolonged for another half hour, after which time the results were read immediately.

To test the inhibition of growth by antisera we used the following procedure. A block of the nutrient agar with a well grown culture was moved, colonies facing downwards, over a fresh medium. In the middle of the streak we put a small disk of filter paper (7 mm diam.) which was previously soaked in undiluted antiserum. After incubation for 2—4 days growth around the disk was examined. In cases where inhibition occurred a ring of two to five mm could be observed macroscopically where growth was absent around the disk in the centre of a well grown culture. Sometimes microscopically a few colonies still could be seen in this ring. The width of the ring varied with the strength of the sera, with the strain and with the height of the layer of nutrient agar in the Petri dish. With negative sera growth continued up to the rim of the disk; sometimes it was even a little heavier here than at some distance.

RESULTS.

1. Complement fixation tests.

The serum prepared with the bovine pleuropneumonia strain A gave positive reactions with the homologous strain and with another bovine strain only. There were no co-reactions with any

TABLE 1.
Complement fixation tests.

Antigens		sera ¹⁾							
		bovine pleuropn.	human oral P.P.L.O.			human urogenital P.P.L.O.			
		A	T	K4	K19	hemol. V26	non hemol. V32 U2 U7		
bovine pleuropn. A		128	—	—	—	—	—	—	—
P2		64	—	—	—	—	—	—	—
P.P.L.O. strains	oral T	—	128	128	128	—	64	64	.
	K4	—	128	64	128	—	—	—	.
	K19	—	128	64	128	—	16	16	64
	K1	—	128	64	128	—	64	64	.
	K2	—	64	64	64	—	32	—	.
	K7	—	128	128	128	—	64	64	.
	K8	—	128	64	128	—	16	16	.
	K11	—	128	32	.	—	32	64	64
	K13	—	128	.	.	—	.	.	.
	K15	—	128	128	128	—	64	64	.
	K18	—	128	128	128	—	16	16	.
	K20	—	128	128	128	—	64	32	.
K25	—	128	128	128	—	64	64	.	
urogenital strains	hemol. V26	—	—	—	—	128	—	—	—
	V38	—	—	—	—	128	—	—	.
	V58	—	—	.	16	64	—	16	.
	G	—	—	—	—	128	—	—	—
	non								
	hemol. V32	—	—	—	—	—	64	64	.
	V37	—	—	—	.	—	64	32	64
	V53	—	—	—	16	—	64	64	.
	V55	—	—	16	.	—	32	64	64
	V65	—	—	—	16	—	64	64	.
	V66	—	—	—	—	—	32	64	64
	V68	—	—	—	16	—	32	32	.
	U2	—	—	—	—	—	16	64	.
	U3	—	—	—	—	—	32	32	64
	U4	—	—	.	—	—	32	32	.
	U5	—	—	—	16	—	—	64	.
	U7	—	—	—	—	—	16	16	64
	V45	—	—	16	.	—	32	32	64

¹⁾ The figures indicate the reciprocal titers giving a ++ reaction: some titers may have been a little higher than 128 but they have not been titrated to the endpoint.

. not tested for lack of antigens.

P.P.L.O. strain antigens; none of the P.P.L.O. sera reacted with bovine strain antigens either. With this method also the hemolytic genital strains could be differentiated from the other P.P.L.O. strains of human origin. There were only a few weak co-reactions with the antigen from one of these strains in other sera. Sera prepared with the oral strains gave strong reactions only with antigens from strains of the same group and showed a few very weak co-reactions with some genital strain antigens. However, sera prepared with the ordinary genital strains were less specific. They gave only weakly positive reactions in approximately the same dilutions with antigens from homologous strains and with the oral strains (see table 1). Control sera and control antigens always gave negative results.

2. Growth inhibition.

We did not succeed in preparing inhibiting sera with all the strains tried. Sera which showed a high titer in the C.F. test sometimes gave no growth inhibition at all. Some sera lost this property on storage rather quickly. Growth inhibition experiments were performed with one serum prepared with a bovine strain, with two sera made with oral ones and with three sera prepared with urogenital strains, two of which were of the hemolytic type.

The results are recorded in table 2. The provisional classification made on the basis of biochemical and morphological behaviour and growth requirements of the various strains is confirmed by the results of the growth inhibition, which on the whole proved to be more specific than the C.F. test. The latter, however, was not at variance with our classification. There are only two strains which still cannot be identified. These are the two strains which grow well in subcultures on not enriched media (U7, V45). In this respect they resemble saprophytic strains. The latter however, grow well at lower temperatures (22—24°C.) which was not the case with our strains U7 and V45. In the C.F. test they cannot be distinguished from the ordinary urogenital strains, but in repeated growth inhibition tests they are not influenced by any one of our sera. Attempts to prepare growth inhibiting sera with these strains were not successful so we cannot answer the question whether they have to be considered as a separate species or not.

TABLE 2.
Growth inhibition by antisera.

Strains	Sera					Classifi- cation	
	Bovine pleuro- pneumonia	P.P.L.O.					
		oral		urogenital			
		T	K19	non hemolytic V 32	hemo- lytic V26 G		
Bovine pleuro- pneumonia A	+	—	—	—	— —	<i>Mycoplasma</i>	
„ P2	+	—	—	—	— —	<i>mycoides</i>	
P.P.L.O. oral strains K4	—	+	+	—	— —	<i>Mycoplasma</i> <i>salivarius</i>	
K11	—	±	+	—	— —		
K14	—	+	+	—	— —		
K19	—	+	+	—	— —		
K23	—	+	+	—	— —		
T	—	+	+	—	— —		
urogenital non hemolytic							
V32	—	—	—	+	— —	<i>Mycoplasma</i> <i>hominis</i>	
V37	—	—	—	+	— —		
V55	—	—	—	+	— —		
V65	—	—	—	+	— —		
U2	—	—	—	+	— —		
U3	—	—	—	+	— —		
U4	—	—	—	+	— —		
urogenital hemolytic							
G	—	—	—	—	+	+	<i>Mycoplasma</i> <i>fermentans</i>
V26	—	—	—	—	+	+	
V38	—	—	—	—	+	+	
V58	—	—	—	—	+	+	
urogenital strains growing without en- richment							
U7	—	—	—	—	— —		
V45	—	—	—	—	— —		

DISCUSSION.

The various P.P.L.O. strains isolated in man can be classified by means of their growth requirements and biochemical and morpholo-

gical behaviour combined with serology. They are easily differentiated from the bovine pleuropneumonia strains (*Mycoplasma mycoides*).

There is a group of oral strains which are regularly found on the buccal mucosa of healthy people and which belong to one species, *Mycoplasma salivarius*. These strains are capable of hemolysing horse blood and do not ferment any sugars. Serologically they are a homogeneous group which in the C.F. test shows some relation with the other human strains; in growth inhibition tests, however, they form a separate group. *M. salivarius* has not been found in connection with pathological conditions. Therefore, provisionally they are considered to be non pathogenic.

Of the urogenital strains from females as well as from males the larger part belong to one species, *Mycoplasma hominis*. In women it was found especially in cases where the normal vaginal flora of Lactobacilli were absent or few in number, which is in agreement with the results of FREUNDT (1953). In half of our male patients with non-specific urethritis P.P.L.O. were cultivated (13 of 26 patients), which is more than is generally found (NICOL and EDWARD, 1953; DUREL *et al.*, 1954; BEEUWKES, 1949). However, the number being small we do not attach too much importance to this difference. Because we did not examine men without genital infections we have no data on healthy people of our own to compare with. Some investigators did not cultivate P.P.L.O. from healthy young men (WAGNER *et al.*, 1953). BOREL obtained positive cultures from 5 out of a group of 28 male controls. NICOL and EDWARD found 10 positives among 90 healthy males three of whom remained positive for at least three months. According to their opinion *M. hominis* is more likely to be a commensal than a pathogen. They suggest, however, that persistence of these microorganisms is favoured by inflammatory conditions.

Neither do our findings allow of our deciding on the significance of the P.P.L.O. in urogenital infections. We hesitate to accept that they are of primary importance mainly because of the fact that we could not see any relation between a positive culture and a certain clinical condition, a fact which is also stressed by NICOL and EDWARD, DUREL *et al.* On the other hand the findings of positive cultures in healthy people are no proof of their innocuity, because certain pathogenic microorganisms can be regular inhabitants of mucous membranes of man, only occasionally causing clinical

symptoms (for example hemolytic streptococci). However, in these cases the clinical picture, if present, is specific for this microorganism.

It looks more probably that the P.P.L.O. may be of secondary importance because of the fact that they are only rarely present in women with a normal vaginal flora; they are often found in females and in males with inflammatory conditions without a specific character. Besides, in male patients with urethritis who were treated with oxytetracycline — to which P.P.L.O. are highly sensitive — symptoms generally subside. In 5 patients whom we could re-examine bacteriologically the cultures became negative after this treatment.

The strains diagnosed as *M. fermentans* differed from the description given by NICOL and EDWARD through their ability to hemolyse horseblood. However, the original G strain of RUITER and WENTHOLT — kindly put at our disposal — was identical in all respects with three of our strains from the vaginal flora of three female patients. In these cases the clinical picture did not show any special feature; in two *Lactobacilli* were scarce, in one they were absent. Discharge was not abundant; in one patient delivery had been 6 weeks previously. There was no association with a fuso-spirochaetal flora as in the case of RUITER and WENTHOLT. Therefore, so far *M. fermentans* can not be considered to be a pathogen of primary importance either. Probably they are secondary invaders and they may have some significance as such.

S u m m a r y.

Serological methods enabled us to confirm the provisional classification which was made on the basis of biochemical behaviour, growth requirements and morphological aspects of P.P.L.O. strains isolated in man.

The growth inhibition test was very specific and allowed of differentiation of nearly all our strains into three species, *Mycoplasma hominis*, *Mycoplasma salivarius* and *Mycoplasma fermentans*. Two strains could not be classified.

The complement fixation test gave on the whole the same results but with this test common antigens were demonstrated in *M. hominis* and *M. salivarius*.

M. salivarius is a common inhabitant of the buccal mucosa of

man and has not been found to have any significance as a pathogen.

M. hominis was found in a large percentage of men suffering from non-specific urethritis and in the vaginal discharge of women where Lactobacilli were absent or scarce.

M. fermentans was isolated from the vagina of three women. They were identical with the G strain of RUITER and WENTHOLT. They were not connected with any special clinical condition. We did not find any relation between a positive culture of *M. hominis* or *M. fermentans* and a specific clinical picture. This makes it improbable that they are real pathogens; maybe they are of secondary importance.

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STUDIES ON TRUE DISSIMILATORY NITRATE REDUCTION

V. NITRIC OXIDE PRODUCTION AND CONSUMPTION BY MICRO-ORGANISMS¹⁾

by

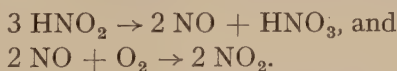
W. VERHOEVEN

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1. INTRODUCTION.

The evolution of NO_2 from fermenting liquids containing nitrate or nitrite has occasionally been reported. Although this phenomenon is often attributed to some microbial activity, observations on the causative organisms as well as on the mechanism of NO_2 formation are rare. Following a recent review of the situation by VERHOEVEN (1952), a number of publications have appeared attesting to a revived interest in the biological production of N-oxides, and indicating novel possibilities for an interpretation of NO and NO_2 production.

VERHOEVEN (1952), studying the appearance of reddish-brown fumes during an industrial alcoholic fermentation of beet molasses, concluded that the production of NO_2 could best be explained as the result of a bacterial reduction of nitrate to nitrite, followed by a purely chemical decomposition of the nitrite in the acid liquid (pH 4.0), as may be represented by the equations:



At that time no clearcut case of microbial NO or NO_2 production in a more direct manner was known. But in a study of *Thiobacillus denitrificans* BAALSRUD and BAALSRUD (1954) showed that under special conditions this organism can provoke not only a qualitative

¹⁾ Part I, II, III and IV: *Antonie van Leeuwenhoek* **20**, 93, 241, 273, 337, 1954.

but even a quantitative conversion of nitrite to NO, and concluded that this is due entirely to a biological process.

Formation of NO was next observed by NAJJAR and ALLEN (1954) during an enzymatic study of denitrification by extracts of *Pseudomonas stutzeri*. These observations have since been extended considerably by CHUNG and NAJJAR (1956a, 1956b).

WYLER and DELWICHE (1954), examining denitrification by soil samples, also noticed NO formation. Because this was pH dependent, they hesitated to conclude that it represented a strictly biological process, and were more inclined to an interpretation along the lines of an acid decomposition of nitrite.

In a general review of chemo-autotrophic bacteria VAN NIEL (1954), commenting on the work of BAALSRUD and BAALSRUD, suggested that their discovery of NO production by *T. denitrificans* from nitrite and thiosulphate might have wider application, and account for the appearance of NO₂ fumes over fermenting liquids even under conditions where a purely chemical decomposition of nitrite by acid would be improbable.

Nevertheless, it seemed possible that even in the experiments of BAALSRUD and BAALSRUD the NO formation was not the result of exclusively biological processes. Gradually several examples of purely chemical nitrite decomposition with NO formation have been described. In the first place KURTENACKER and SPIELHACZEK (1934) have demonstrated NO evolution as a purely chemical event from a mixture of thiosulphate and nitrite, and these are precisely the only substances from which the BAALSRUDS obtained NO in their experiments with resting cell suspensions of *T. denitrificans*. Besides, in the latter case NO continues to be produced in the presence of HgCl₂ in concentrations that generally prevent all biological activity; it can be arrested only when the concentration of the poison is raised to excessively high levels (1%)!

The French workers LEMOIGNE, MONGUILLON and DESVEAUX (1937) obtained purely chemical reactions between nitrite and ascorbic acid with formation of NO at room temperature. In this case, however, the medium was more acid than usually encountered in denitrification (pH 4.0).

These observations are quite recently confirmed and considerably extended by the work of EVANS and MCAULIFFE (1956). They could even show that reduced T.P.N. also reacted with nitrite yielding NO.

Consequently some further studies on NO production seemed in order. They are here reported, and comprise an investigation of the chemical decomposition of nitrite in the absence and presence of thiosulphate in solutions of different acidity; of the reaction induced by *Thiobacillus*; and of the formation of NO by other denitrifying bacteria, including the potentially autotrophic *Micrococcus denitrificans* and some heterotrophic denitrifiers. Finally, this study was extended to observations on metabolic processes in which NO itself can function as an oxidant.

2. CHEMICAL DECOMPOSITION OF NITRITE.

The decomposition of nitrite by acid was studied with the conventional manometric techniques. For this purpose 2 ml of an acetic acid-acetate (0.2 M) or phosphate buffer (0.25 M) were pipetted into the main compartment, and 0.1 ml of a 0.2 M nitrite solution was placed in the side arm of a Warburg flask. In the experiments with thiosulphate and nitrite 0.1 ml of 0.1 M thiosulphate was added to the contents of the main compartment. The air in the manometers and vessels was replaced by nitrogen, and after temperature equilibration the contents of the side arm were tipped in. Gas evolution commenced at once, especially at lower pH. The results of the experiments, expressed as μl gas formed, are given in table 1.

TABLE 1.

Chemical decomposition of nitrite at different pH in μl gas in absence and presence of thiosulphate

	pH									
	3.4	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0
Gas evolution from $20\mu\text{ mol NO}_2$ during 60 min.	63	42		43	9	9	6	2	—	—
Gas evolution from $20\mu\text{ mol NO}_2'$ and $20\mu\text{ mol S}_2\text{O}_3''$ during 40 min.		246	234	189	91	27	21	9	3	

The data show that relatively large amounts of gas are formed. It can scarcely be doubted that in the case of the acid decomposition of nitrite the gas was NO. In the reaction between thiosulphate and

nitrite it is probable that a mixture of N_2O and NO was produced.

That in both cases NO was indeed present could be shown with the aid of the KOH -sulphite technique used by the BAALSRUDS. It can be concluded that the NO -production, as observed by VERHOEVEN (1952) and by WYLER and DELWICHE (1954), is explainable on the basis of a purely chemical decomposition of nitrite.

The observation of the BAALSRUDS cannot, however, be accounted for in this manner because they observed a rapid and quantitative nitrite decomposition to NO by *Thiobacillus* suspensions at pH 6.4, where the chemical reaction between nitrite and thiosulphate is negligible.

3. PRODUCTION OF NO BY *Thiobacillus denitrificans*.

These results imply that the formation of NO by *T. denitrificans* is an indubitably biological process. They led to a more detailed study, especially with a view to ascertaining whether NO could be produced by this organism even in neutral media.

The cells were grown in the liquid medium with thiosulphate and nitrate devised by BAALSRUD and BAALSRUD, harvested by centrifugation, and suspended in 0.05 M phosphate buffer (BAALSRUD and BAALSRUD, 1954). To determine NO , duplicate experiments were run in the presence of KOH -sulphite, and of KOH , respectively, in the center well. The results of these experiments are reported graphically in fig. 1.

As can be seen, NO evolution at pH 6.4 occurs as described by the BAALSRUDS. The maximum amount of gas liberated from 10 μ M nitrite is well above the amount to be expected on the assumption that the nitrite is converted into a gaseous compound with two nitrogen atoms (10 μ M nitrite can yield 112 μ l N_2O or N_2 , and 224 μ l NO). Together with the observation that a considerable proportion of the gas is absorbed by alkaline sulphite, this indicates that, indeed, large quantities of NO are produced. The subsequent disappearance of gas in the experiment with KOH in the center well shows the secondary NO consumption earlier noticed by the BAALSRUDS.

Although the biological formation of NO at pH 6.4 had thus been fully confirmed, it is evident from Fig. 1 that at pH 7.2 the result is quite different; here the total amount of gas formed from the added nitrite is close to the theoretical amount of 112 μ l;

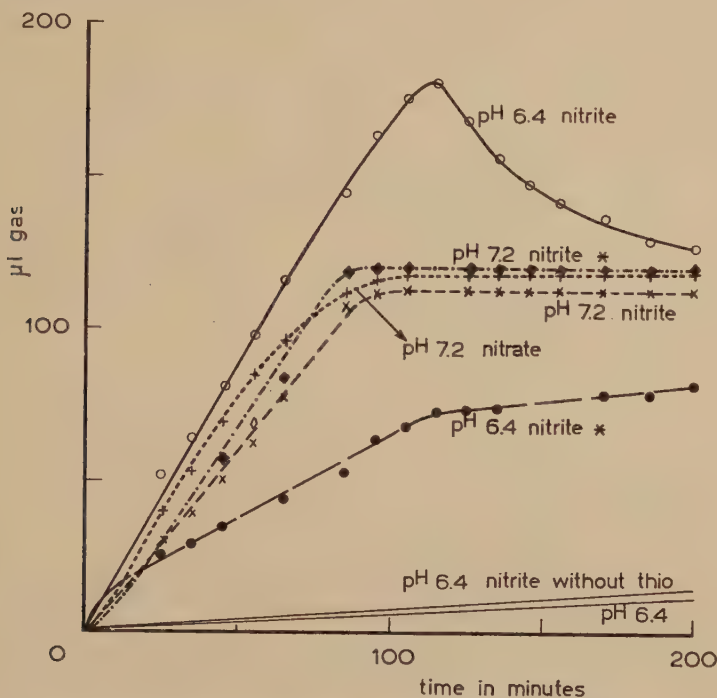


Fig. 1. 3 day-old cells of *Thiobacillus denitrificans* suspended in phosphate buffer, pH 6.4, or 7.2. In all vessels 20μ moles of thiosulphate are present unless otherwise indicated. Additions of 10μ moles of nitrate or nitrite are shown in the figure. The curves pertaining to experiments with a 10% KOH, 10% sulphite mixture in the center well are marked with *.

an equal quantity is liberated from nitrate. Furthermore, in the experiment with KOH—sulphite in the center well the same amount of gas is produced as in the experiment with KOH only, demonstrating the absence of NO among the gaseous reaction products. It is therefore clear that NO production is restricted to special conditions.

The surprisingly low inhibiting effect of HgCl_2 led to some experiments on the effect of cyanide, a well-known inhibitor of denitrification, on nitrite decomposition by *T. denitrificans*.

The cells were suspended in a phosphate buffer at pH 6.4, and 2 ml of the suspension pipetted into the main vessel together with 0.1 ml of a cyanide solution of appropriate concentration, previously adjusted to pH 6.4. The side arm contained $10\mu\text{M}$ of nitrite and $20\mu\text{M}$ of thiosulphate. The outcome of these experiments is given in fig. 2.

As is illustrated, the presence of cyanide has a very pronounced

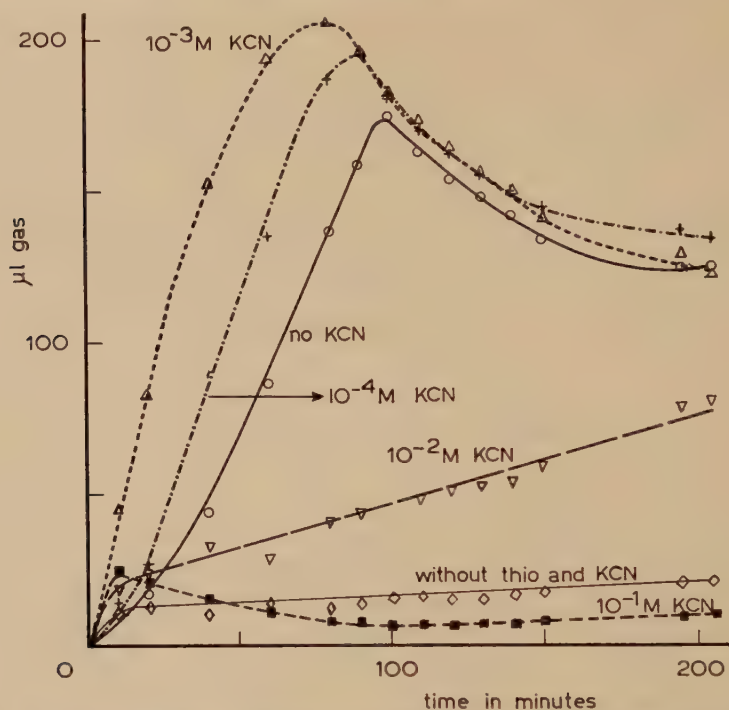


Fig. 2. 3 day-old cells of *Thiobacillus denitrificans* suspended in phosphate buffer, pH 6.4, with 10μ moles nitrite and 20μ moles thiosulphate. Final KCN concentrations indicated in figure.

influence. In the experiment without cyanide the usual NO production is noticed. In the presence of 10^{-4} and 10^{-3} M cyanide the total amount of gas evolved is still greater, implying an enhanced NO production.

The failure of cyanide at these concentrations to prevent nitrite reduction is in keeping with previous information (ALLEN and VAN NIEL, 1952), as is also the pronounced inhibition by the higher concentrations. The results of this experiment can thus be taken as a further confirmation of the biological origin of the NO.

The increased NO production from nitrite in the presence of low cyanide concentrations suggested the possibility that under such conditions NO might also be formed from nitrate. Appropriate experiments, represented in fig. 3, have shown that this is not the case. The results simply indicate a general inhibitory effect on the denitrification reaction. The greater sensitivity of nitrate than of

nitrite reduction towards cyanide, previously noticed in other studies on denitrification (ALLEN and VAN NIEL, 1952), is thus corroborated also for *T. denitrificans*.

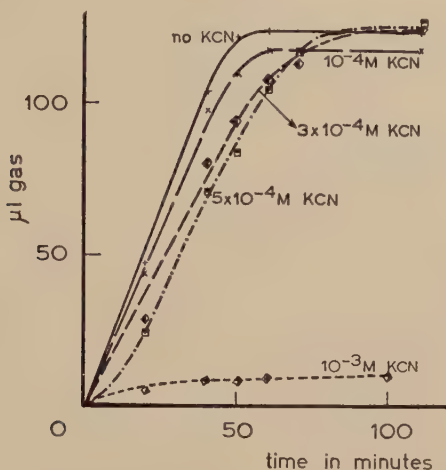


Fig. 3. 3 day-old cells of *Thiobacillus denitrificans* suspended in phosphate buffer, pH 6.4 with 10μ moles nitrate and 20μ moles thiosulphate. Final KCN concentrations indicated in figure.

Consequently at pH 6.4 the fate of nitrite is quite different from that of nitrate. It is important to realize that these two oxidants also affect the bacteria differently. As found by the BAALSRUDS, nitrite is usually as toxic to *T. denitrificans* as is cyanide; only in the presence of thiosulphate does this toxicity not result in the cessation but in a modification of the reduction process, now yielding the unusual reduction product, NO. This suggested that the formation of NO may be caused more generally by physiologically unfavorable conditions.

This is further supported by the results of experiments on the occurrence of NO as a product of nitrite reduction at pH 7.2. Initial experiments with suspensions of cells harvested from 3-day old cultures showed that even in the presence of cyanide the production of NO could not be detected. However, a surprising observation was made when a 10-day old culture was used; these cells could be induced to evolve NO also in a neutral medium with appropriate cyanide concentrations, as is shown in fig. 4.

This figure presents some interesting features, especially if it is

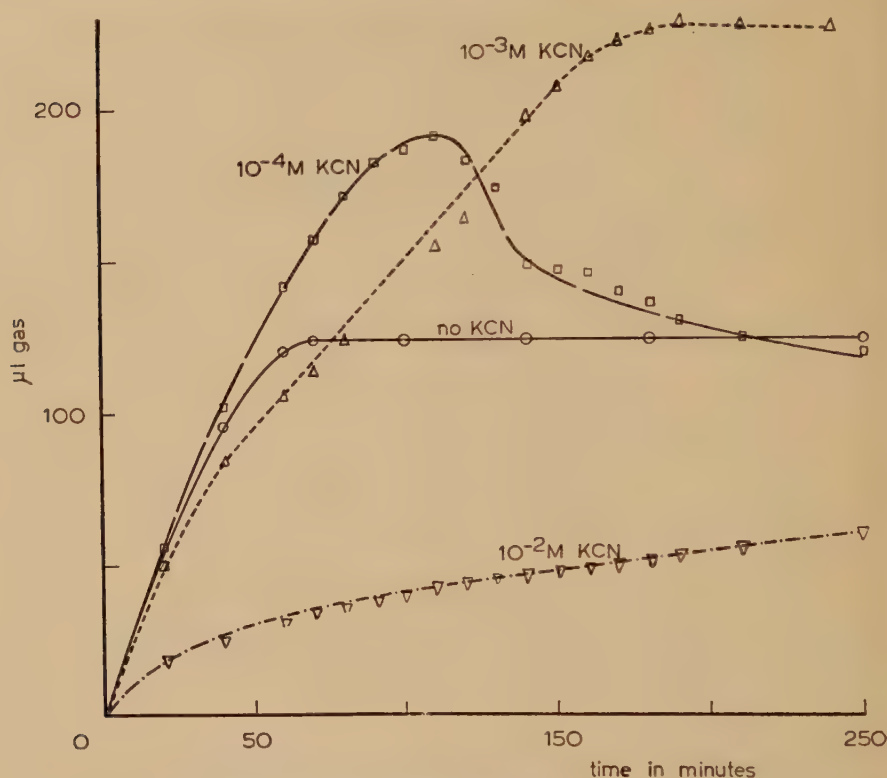


Fig. 4. 10 day-old cells of *Thiobacillus denitrificans* suspended in phosphate buffer, pH 7.2, with 10μ moles nitrite and 20μ moles thiosulphate. Final KCN concentrations indicated in figure.

compared with fig. 2. It shows that NO formation is now strictly dependent on the presence of cyanide. In line with the previous reasoning this can be ascribed to the fact that at pH 7.2 nitrite itself is far less toxic than at pH 6.4. Besides, the secondary utilization of NO at pH 7.2 occurs only at the lowest cyanide concentration. At a concentration of 10^{-3} M, where the "young" cells still reduce NO at pH 6.4, the "old" cells no longer carry out this reaction; the final amount of NO evolved agrees with the theoretically obtainable maximum.

All the experimental results described in this section thus fit in with the notion that NO production by *Thiobacillus* occurs only under adverse conditions. "Physiologically old" cells and/or the presence of toxic substances appear to be requisites.

4. PRODUCTION OF NO BY HETEROTROPHIC DENITRIFYING BACTERIA.

Once the reality of NO formation by *Thiobacillus* as a biological process had been established beyond reasonable doubt, it was of interest to determine whether NO production could also be demonstrated with other denitrifying bacteria. Because thus far NO had been found only as the result of a partial oxidation of thiosulphate with nitrite as oxidant, the first experiments were carried out with denitrifying bacteria that are known to be able to oxidize thiosulphate. As such, *Micrococcus denitrificans* and *Pseudomonas stutzeri* were used (SIJDERIUS, 1946).

Because the growth of these bacteria in media with thiosulphate as the sole hydrogen donator is at best very poor, *P. stutzeri* (strain ALLEN and VAN NIEL, 1952) was cultivated in stoppered bottles with 5% yeast autolysate, 0.1% K_2HPO_4 , 0.05% $MgSO_4 \cdot 6aq$, 0.5% KNO_3 , 0.5% $Na_2S_2O_3 \cdot 5aq$; pH 6.8, while for *M. denitrificans* (strain BEIJERINCK) the same medium with 1% KNO_3 was used.

The harvested cells were resuspended in phosphate buffer, pH 6.4, and 2 ml of the suspension pipetted into the main compartment together with the requisite amount of cyanide: 10μ M of nitrite and

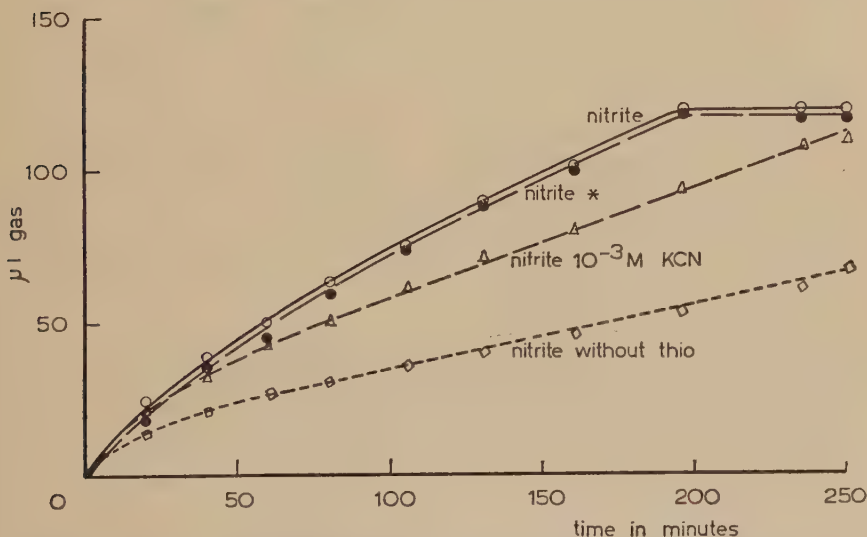


Fig. 5. Cells of *Pseudomonas stutzeri* suspended in phosphate buffer, pH 6.4. All vessels contained 20μ moles of thiosulphate unless otherwise indicated. Additions of 10μ moles of nitrite or nitrate and the final molarity of the added cyanide shown in the figure. The curve pertaining to the experiment with a 10% KOH, 10% sulphite in the center well is marked with *.

20 μ M of thiosulphate were added from the side arm after equilibration. The results of the experiment with *P. stutzeri* are given in fig. 5, with *M. denitrificans* in fig. 6.

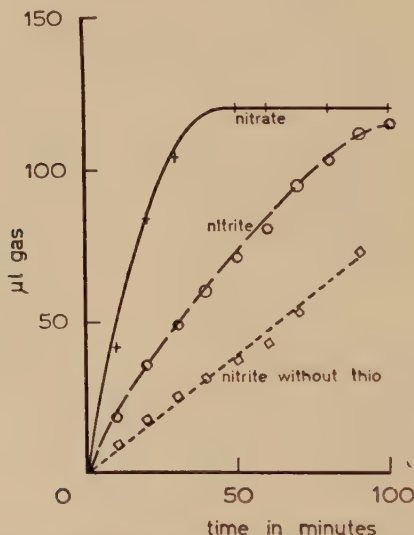


Fig. 6. Cells of *Micrococcus denitrificans* suspended in phosphate buffer, pH 6.4. All vessels contained 20 μ moles of thiosulphate unless otherwise indicated. Additions of 10 μ moles of nitrate or nitrite shown in the figure.

These figures show that neither *M. denitrificans* nor *P. stutzeri* produce NO under conditions that cause *Thiobacillus* to do so. Of interest is also the fact that the rate of gas production by *P. stutzeri* is inhibited by 10^{-3} M KCN, which would not be expected if NO formation were to replace the normal N_2 evolution (compare, *e.g.*, Fig. 2).

Nevertheless, the NO production by enzyme extracts of *P. stutzeri* observed by NAJJAR and ALLEN (1954) showed that heterotrophic denitrifiers are potentially capable of liberating NO from nitrite. For this reason many experiments were carried out to determine whether whole cells of such organisms, supplied with organic substrates and nitrite, could yield NO. Initially the results were invariably negative, which is in complete agreement with many previous studies. It was, however, possible to show NO production by enzyme extracts of *P. aeruginosa* and *M. denitrificans* thus extending the observations of NAJJAR and ALLEN.

But after it had been found (section 3) that old cultures of

Thiobacillus can form NO under conditions where young cultures merely produce N_2 , a more detailed study was made of the effect of physiological age on the metabolic products of heterotrophic denitrifiers. The first experiments were carried out with *P. aeruginosa*; later, *M. denitrificans* was also included in this study.

The bacteria were cultivated anaerobically in a medium composed of 0.2% yeast autolysate, 1% KNO_3 , and 0.5% glucose, pH 6.8, and growth was measured turbidimetrically in a Klett-colorimeter as a function of time. These observations showed that growth ceased after 22 hours. At different times samples were removed, the cells harvested, and resuspended in phosphate buffer at pH 6.4 and 7.2. The suspensions were always standardized to the same density.

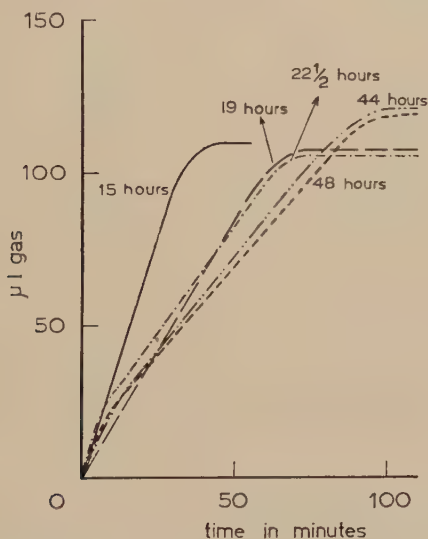


Fig. 7. Cells of *Pseudomonas aeruginosa* of different age suspended in phosphate buffer, pH 6.4, with 10μ moles of nitrite and 10μ moles of glucose. The age of the cells is shown in the figure. Similar experiments with a 10% KOH, 10% sulphite mixture in the center well gave identical results; those experiments with nitrate instead of nitrite were also similar.

In every experiment the gas production from glucose and nitrate or nitrite was measured at the two pH values, and simultaneously with KOH and alkaline sulphite, respectively, in the center well. As shown in Fig. 7, NO was not evolved from nitrite by any of the suspensions. Except for a difference in the rate of gas production (ratio of velocities with nitrate and nitrite, respectively, equal to

3 : 5, in agreement with many previous investigations), the behavior of the cells towards nitrate was identical.

At first sight the high rate of denitrification of the 15-hour old culture may be somewhat surprising, since it has been stated that growth continued for 22 hours, so that one might have expected a uniform behavior of the suspensions derived from cultures up to this age. The explanation of this behavior is, however, quite simple; due to the pronounced liberation of alkali during the denitrification process, the culture conditions become progressively more unfavorable as soon as the buffer capacity of the medium has been exceeded. Corroborating this explanation is the fact that growth is not exponential throughout the period during which the cell mass increases. Consequently it is reasonable to ascribe the high activity of the cells from the 15-hour old culture to the fact that at that time the environment had not yet become unfavorable.

Although the cells from increasingly older cultures display the same activity under the conditions employed, the picture changes drastically if the experiment is performed with the addition of 10^{-3} M cyanide. The results of such experiments, at pH 6.4, are shown in fig. 8.

It will be seen that cells from young cultures are merely inhibited by the poison; but those from 44 and 48-hour old cultures now produce copious amounts of NO.

At pH 7.2 NO formation was not observed, even in the presence of cyanide: nor was it ever found in experiments with nitrate as the oxidant. Entirely comparable results were obtained with cultures of *M. denitrificans*.

These results demonstrate that heterotrophic denitrifiers can indeed produce NO from nitrite. It seems difficult to ascribe this NO formation merely to a chemical reaction between reduced T.P.N. and nitrite (EVANS and MCAULIFFE, 1956), since it is also evident that, as in the case of *Thiobacillus*, NO production happens only under adverse conditions. For the latter organism nitrite itself is a strong poison (BAALSRUD and BAALSRUD, 1954); for other denitrifiers that normally oxidize organic substrates it is generally much less inhibitory. An exceptional situation is that of *M. denitrificans*, for which nitrite is highly toxic if the organism must depend on the oxidation of H_2 , but harmless, if organic H-donors are used (KLUYVER and VERHOEVEN, 1954). That the heterotrophic denitrifiers produce NO from nitrite only in the presence of an additional

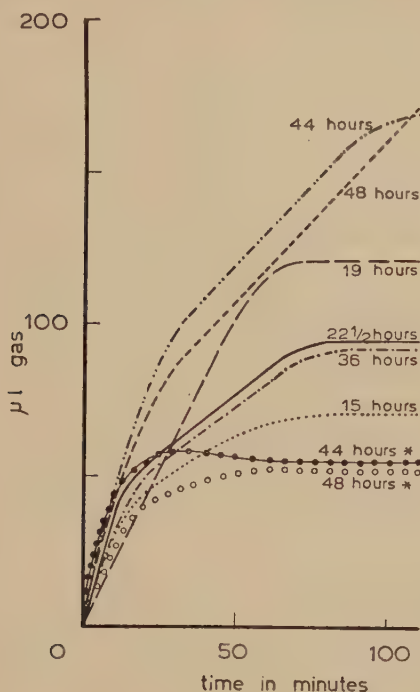


Fig. 8. Cells of *Pseudomonas aeruginosa* of different age suspended in phosphate buffer, pH 6.4, with 10μ moles each of nitrite and of glucose and cyanide to a final concentration of $10^{-3}M$. The age of the cells is shown in the figure. The data referring to experiments of 44 and 48 hour-old cells in which a 10% KOH, 10% sulphite mixture was placed in the center well are marked with *. The results with younger cells with KOH sulphite mixture were identical with those without sulphite.

toxic agent thus emphasises the need of an unfavorable environment in order to induce NO formation. Besides, the physiological age of the cells obviously plays an important part; but an explanation of this gerontological phenomenon cannot yet be attempted.

5. CONSUMPTION OF NO BY MICRO-ORGANISMS.

These developments naturally led to the question whether the observed formation of NO could be interpreted as implicating this substance as a more or less normal intermediate product in denitrification. The fact that such utterly unphysiological conditions are needed to detect NO evolution should warn against too ready an acceptance of this possibility, although the positive outcome of experiments with enzyme extracts is perhaps suggestive.

If NO were a normal intermediate product, it should be reducible to N_2O and N_2 by all denitrifying bacteria. BAALSRUD and BAALSRUD observed that *T. denitrificans* actually converts NO to gaseous products with 2 N atoms per molecule; this has been fully confirmed (section 3). But the behavior of other denitrifiers in this respect was unknown, and hence this point was further investigated with *M. denitrificans* and *P. stutzeri*. The bacteria were cultivated under anaerobic conditions in a 10% yeast autolysate, $\frac{1}{2}\%$ KNO_3 , pH 6.8 medium. After harvesting the bacteria were suspended in a 0.05 M phosphate buffer at pH 6.8. The cell suspension was placed in Warburg cups containing KOH in the center well and 20μ M of glucose in the side arm.

After flushing with nitrogen the gas phase was replaced with N_2 -NO mixtures of known composition. Some of the results of the experiments with *P. stutzeri* are reported in figure 9.

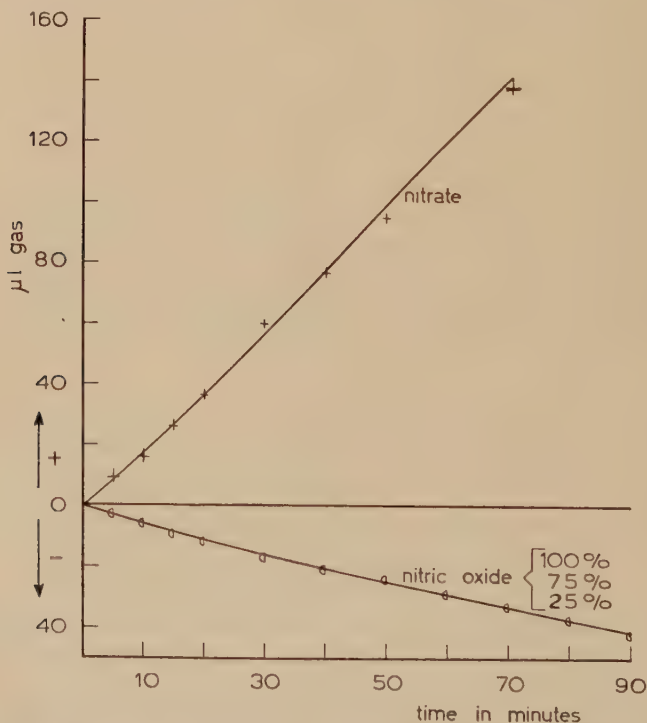
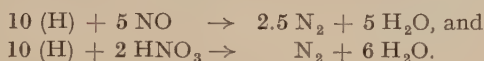


Fig. 9. Cells of *Pseudomonas stutzeri* suspended in phosphate buffer, pH 6.4, with 10μ moles of glucose. In the vessel with nitrate 10μ moles added, the atmosphere in experiments with NO consisted of 100% NO; 75% NO - 25% N_2 ; and 25% NO - 75% N_2 , respectively.

The figure shows that *P. stutzeri* consumes NO in an atmosphere of 100%, 75% and 25% NO.

With *M. denitrificans* essentially the same behavior was observed.

From the slope of the curve for gas production from nitrate it follows that the suspension produced 120 μ l N₂ per hour; the slope of the curve representing gas consumption in the experiment with NO permits the calculation that here 30 μ l N₂ are formed per hour. In previous experiments with denitrifying bacteria it has generally been found, that the rate of N₂ production is determined by the capacity of the dehydrogenases (VAN OLDEN, 1940; ALLEN and VAN NIEL, 1952; KLUYVER and VERHOEVEN, 1954). Were this is true also for the reduction of NO, the rate of N₂ evolution from NO should be 2.5 times that from nitrate, according to the equations:



The observed rates bear the ratio of 1 : 4, however, this means that the enzymatic capacity for reducing NO falls far below that required, and indicates that NO may not be a normal intermediate product in denitrification, a contention that is strengthened by the earlier discussed conditions needed for obtaining NO production.

The mere fact that denitrifying bacteria can reduce NO does not necessarily implicate this substance as a normal intermediate product. There are many instances of reductive processes, collectively designated by NEUBERG as "phytochemical reductions" (NEUBERG, 1920), that are at best distantly related to the normal metabolic pattern of an organism, and the reduction of NO, like that of methylene blue, for example, might belong to this category. In that event organisms that are known not to be denitrifiers might exhibit the same property.

From the studies of MEYERHOF and SCHULZ (1935) it is known that yeast maceration juice can reduce NO. This observation was somewhat extended by experiments with intact cells of yeast (*Saccharomyces cerevisiae*) and of a non-denitrifying *P. fluorescens*. The former were grown in distilled water with 0.1% KH₂PO₄, 0.05% MgSO₄·5H₂O, 0.1% NH₄Cl, 1% glucose, and a few drops of yeast autolysate; the latter in distilled water with 0.1% KH₂PO₄, 0.05% MgSO₄·5 H₂O, and 1% NH₄-succinate (pH 6.8).

After harvesting and suspending the micro-organisms in phosphate buffer at pH 6.8, manometric experiments were conducted

with either air or NO as gas phase. In the case of *P. fluorescens* succinate was used as hydrogen donator, for *S. cerevisiae* glucose or acetate served as such.

Figure 10 illustrates the gas consumption by cell suspensions of aerobically grown *P. fluorescens* in NO and in air. On the assumption that the NO is converted into N_2 , the NO consumption is twice the observed gas exchange: this lags far behind the oxygen uptake.

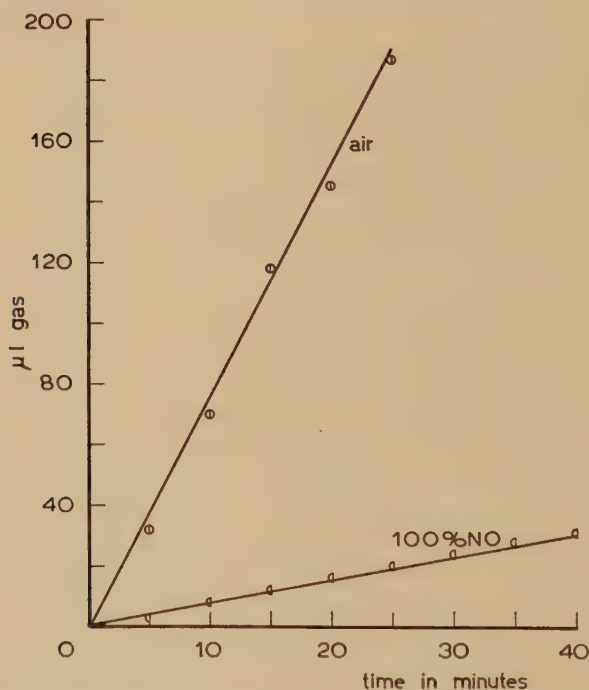


Fig. 10. Cells of *Pseudomonas fluorescens* grown aerobically in the absence of nitrate, suspended in phosphate buffer, pH 6.8, with 20μ moles of succinate. Atmosphere: air or 100% NO.

The manometric experiments with *S. cerevisiae* gave essentially similar results. These findings show that organisms, devoid of denitrifying ability, and not previously exposed to NO, can indeed reduce this substance, although at a relatively low rate. This is generally true for phytochemical reductions.

In view of the special properties of NO it was considered possible that this gas might enter into a purely chemical reaction with some cellular constituent. From the work of KEILIN and HARTREE (1937)

it is known that cytochrome can form a complex with NO, so that there was reason to investigate this possibility.

VERHOEVEN and TAKEDA (1956) have found that from *P. aeruginosa* an enzyme extract can be prepared which in a reduced condition displays the characteristic absorption bands of Fe^{++} -cytochrome. Upon admission of NO to such an extract a shift in the absorption characteristics can be observed compatible with a partial oxidation of the cytochrome.

Following this observation a cytochrome pigment was isolated by treatment with citric acid (VERHOEVEN and TAKEDA, 1956). After reduction with $\text{Na}_2\text{S}_2\text{O}_4$ a solution of this cytochrome was transferred to a Thunberg tube to which a Beckman cuvette was attached, and the Thunberg tube repeatedly evacuated and flushed with nitrogen. The absorption spectrum of the solution was determined between 500 and 600 $\text{m}\mu$. A second absorption spectrum of the solution was measured after admission of NO to the tube. The results are shown in fig. 11.

As can be seen the absorption spectrum of the reduced pigment changes appreciably after the addition of NO, and becomes identical with that of the oxidized cytochrome. This observation shows that NO can oxidize the reduced cytochrome; it was, however, surprising that the characteristic cytochrome-NO complex did not seem to be formed.

An explanation of this situation was provided by the discovery that, upon opening the Thunberg tube containing NO, the solution showed a marked color change. A measurement of the absorption spectrum showed that now, indeed, KEILIN's cytochrome-NO complex was formed with the typical absorption peaks at 530 and 560 $\text{m}\mu$.

This suggested that the NO_2 formed after opening the tube had reacted with the cytochrome and modified its properties. In that event the observations of KEILIN and HARTREE would have to be ascribed to the use of a cytochrome preparation with initial characters unlike those of our preparation. It should here be emphasized that KEILIN had obtained his cytochrome by a procedure involving treatment with trichloroacetic acid (TCA), while our sample had been extracted with citric acid. In our experience (VERHOEVEN and TAKEDA, 1956) cytochromes prepared by these different extraction procedures can behave in a strikingly dissimilar manner.

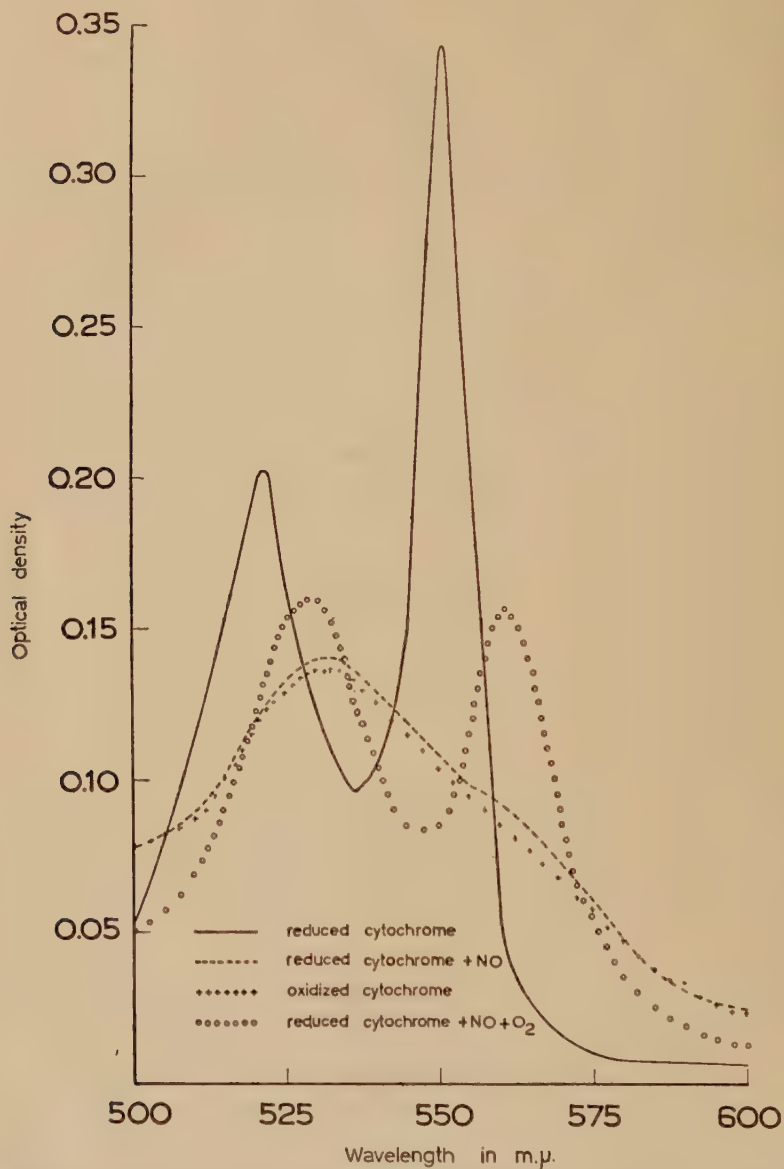


Fig. 11. Absorption spectra of isolated *Pseudomonas aeruginosa* cytochrome in the reduced and oxidized form, and after reaction of reduced cytochrome with NO and NO + O₂.

Consequently this possibility was tested with a sample of *P. aeruginosa* cytochrome, extracted with citric acid. A solution of this preparation was divided into two parts; one aliquot served as control, while the other was subjected to an additional treatment with TCA according to the procedure of VERNON and KAMEN (1954). The absorption spectra of the two solutions, made up so as to cause identical absorption at 552 μ , were determined under a variety of conditions; the results are represented in fig. 12.

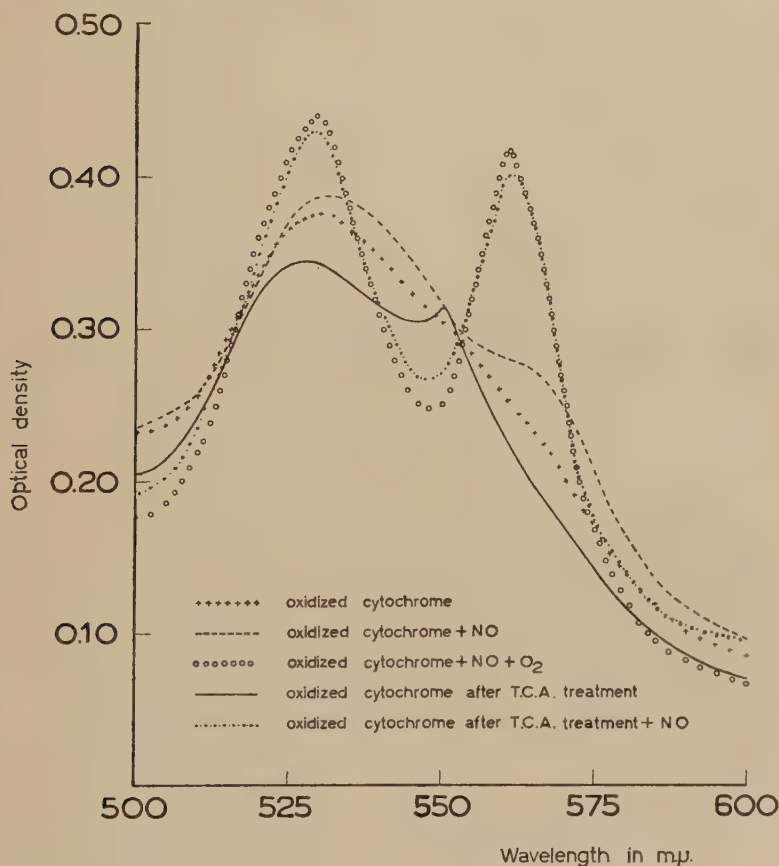


Fig. 12. Absorption spectra of isolated *Pseudomonas aeruginosa* cytochrome before and after treatment with trichloroacetic acid in the oxidized form and reaction with NO.

It is immediately apparent that TCA treatment had modified the properties of the cytochrome. Whereas, in agreement with our previous observations, the initial cytochrome in the oxidized state

was not affected by NO, but readily formed the NO-complex when both NO and O₂ were admitted, the TCA-treated sample behaved in the manner described by KEILIN and HARTREE, and yielded the complex immediately upon exposure of the solution to pure NO. Samples of commercial mammalian cytochrome C as well as samples of cytochrome prepared from beef heart with the TCA method also produced the typical complex immediately with NO in the absence of O₂.

It seems reasonable to assume that the properties of native cytochrome are approximated more closely by those of the pigment extracted with citric acid than by those of the product obtained by TCA treatment. The strongest argument in favor of this contention is that enzyme extracts, too, did not yield an NO-complex when exposed to NO in the absence of O₂. The following considerations are based upon this assumption.

It has been shown that NO can oxidize the reduced form of the cytochrome. It may thus be concluded that the uptake of NO by cell suspensions should be a general property of all organisms that contain cytochrome. It will then also be evident that the observed NO consumption by denitrifying bacteria cannot be used as a decisive argument in determining whether or not this property implicates NO as a normal intermediate product in denitrification.

6. IS NO A NORMAL INTERMEDIATE PRODUCT IN DENITRIFICATION?

The production of NO by whole cells and cell-free extracts of denitrifying bacteria could be interpreted to mean that this substance represents a normal intermediate stage in the reduction of nitrate to N₂. The fact that only under decidedly adverse conditions accumulation of NO is readily demonstrable is not, of course, a convincing argument against this supposition. Typical intermediate products do not normally accumulate: during undisturbed metabolism they are not apt to be present in more than trace amounts. Only if conditions are imposed that prevent those metabolic processes concerned with the further conversion of an intermediate product to proceed at their normal rate can accumulation be induced. The need for adverse conditions to cause NO production could be thus understood; similarly NO formation by cell-free extracts (NAJJAR and ALLEN, CHUNG and NAJJAR) might signify that the relative activities of various enzymes had become grossly distorted as a result of the extraction procedure.

On the other hand, the fact that a genuine intermediate product does not ordinarily accumulate, precisely because subsequent conversions proceed at a commensurate rate, implies that it should be metabolized at least as rapidly as its precursors. But then the low rate at which denitrifying bacteria reduce NO as compared with nitrate and nitrite argues against NO being such an intermediate product.

It must be granted that one can formulate subsidiary hypotheses to reconcile the observed rate of NO reduction with the intermediate product concept. Access of the gas to enzyme sites, establishment of appropriate concentrations for optimum enzyme activity, and possible inhibitory effects of NO at the concentrations used in the present experiments may be advanced as such. It could even be proposed that it is not NO itself but a particular complex with some other substance that is the "true" intermediate product. In the absence of decisive experiments such reasoning becomes, however, rather futile and largely a matter of semantics. This was also the opinion voiced during the discussions at the symposium on inorganic nitrogen metabolism (Baltimore, 1956; espec. pp. 280 and 286). For this reason the problem will not be further pursued here.

S u m m a r y.

A detailed study of the conditions of NO formation by *Thiobacillus* suspensions led to the conclusion that NO occurs only under circumstances that are decidedly adverse to normal metabolism. The presence of otherwise toxic concentrations of nitrite at pH 6.4, of cyanide or of a combination of both can be mentioned.

A striking observation was that even at pH 7.2 "physiologically old" cells did show NO formation, but then also only in the presence of nitrite and cyanide.

It could be shown that the thiosulphate oxidizing bacteria *Micrococcus denitrificans* and *Pseudomonas stutzeri* evolved no NO under conditions in which *T. denitrificans* did.

The typical influence of the "physiological age" of the cells in the experiments with *T. denitrificans* suggested the possibility of NO production with "old" cells of *Ps. aeruginosa* grown on nitrate and glucose.

With these "physiologically old" bacteria NO production could indeed be obtained. The adverse conditions, already so characterist-

ic in the experiments with *T. denitrificans*, were still more pronounced in this case. Here even the combined conditions of low pH (6.4), presence of nitrite and cyanide and "physiologically old" cells were necessary for the production of this gas.

The consumption of NO by *Pseudomonas stutzeri* and *Micrococcus denitrificans* is established. However, suspensions of the non denitrifying microorganisms *Pseudomonas fluorescens* and *Saccharomyces cerevisiae* showed also NO consumption when cultivated aerobically in the absence of nitrate. The significance of NO production and consumption by microorganisms is discussed, together with some observations on the behaviour of isolated bacterial cytochrome towards NO.

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CAUSES OF DEATH OF BACTERIA IN FROZEN SUSPENSIONS¹⁾

by

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(Received July 21, 1956).

INTRODUCTION.

It is sometimes asserted that death of bacteria in frozen suspensions is due to ice formation *per se*, either by direct crushing by intracellular or extracellular ice crystals or by pressure resulting from ice formation (KEITH, 1913; HILLIARD and DAVIS, 1918; YOUNG, 1929; BERRY, 1933; WEISER and OSTERUD, 1945; PROOM, 1952; HOLLANDER and NELL, 1954). On the other hand, certain observations would indicate that some factor other than ice formation *per se* is responsible (PRUDDEN, 1887; MACFAYDEN and ROWLAND, 1900; DUBOS, 1937; HAINES, 1938; TURNER and BRAYTON, 1939; CICCONI, 1941). Moreover, it has been demonstrated (HARRISON and CERRONI, 1956) that there is no correlation between the physical strength of bacterial cells and their susceptibility to the lethal effect of freezing and thawing. Therefore, it is unlikely that the lethal factor inherent in freezing-thawing is mechanical. The present paper will demonstrate similarities between the behavior of bacteria in frozen suspensions at -22°C . and in unsolidified suspensions at this same temperature. From this, conclusions will be formulated to account for death in frozen suspension.

MATERIALS AND METHODS.

Escherichia coli strain 69L-15, *Serratia marcescens* strain ATCC 274, and *Lactobacillus fermenti* strain 69L-3 were employed as test

¹⁾ This research was supported in part by funds provided by the Vanderbilt University Research Council.

organisms. They were cultivated as already outlined (MAJOR, *et al.*, 1955). The broth cultures were centrifuged at $2,000 \times G$ for 30 minutes; the cells were washed twice with distilled water, were finally suspended in distilled water, and then aliquots of this suspension were added to various suspending media. Five ml aliquots of the test suspension were dispensed into test tubes (15 mm by 150 mm) held in a water bath at 4°C ., the tubes were plugged with rubber stoppers and then were transferred to an ethyl alcohol bath at -22°C . After the tubes had attained this temperature they were removed from the alcohol bath but were kept within the cold chest at $-22 \pm 2^{\circ}\text{C}$.

Unsolidified suspensions were sampled by transferring a 0.1 ml aliquot directly to a dilution bottle containing diluent at room temperature. Solidified suspensions were thawed (35°C .) and then sampled in like manner. The composition of the diluent and the dilution and plating techniques already have been given (MAJOR *et al.*, 1955; HARRISON, 1955).

RESULTS AND DISCUSSION.

Effect of solute upon survival. In order to determine the effect of solute upon survival, cells were frozen in distilled water and in various concentrations of broth. The data in table 1 illustrate that survival of *Escherichia coli* is improved by decreasing the amount of solute present, survival being best in the total absence of added solute — in distilled water. *Serratia marcescens*, not included in the table, also survives best in the absence of added solute. In the case of *Lactobacillus fermenti*, survival is poorer in broth diluted 10-fold, but it improves as the broth is diluted further and, as with *E. coli* and *S. marcescens*, is best when no solute is present.

If crushing by ice were the primary lethal factor, a behavior just the reverse of that in table 1 would be expected. At a temperature above the eutectic point of the solute, water will be removed from the suspending medium in the form of ice and the cells will then reside in unsolidified solute. At lower concentrations of broth there would be less unsolidified solute in which the cells could reside and thereby escape crushing. Hence, survival should be minimal in distilled water. Actually, at initial viable cell counts above 10^7 per ml survival is maximal in distilled water.

If the behavior of bacteria in frozen suspensions is not due to ice

TABLE 1.

Survival of bacteria in different concentrations of solute at -22°C .*Escherichia coli*Initial viable cell count per ml: 1.4×10^8

Menstruum	Viable cell count per ml after storage for:		
	1 day	3 days	1 week
Undiluted broth ¹⁾	1.4×10^7	1.9×10^6	3.2×10^5
Broth diluted 10-fold	3.6×10^7	9.8×10^6	4.5×10^6
Broth diluted 100-fold	4.0×10^7	3.2×10^7	3.7×10^7
Distilled water	1.2×10^8	9.0×10^7	9.6×10^7

*Lactobacillus fermenti*Initial viable cell count per ml: 5.6×10^7

Menstruum	Viable cell count per ml after storage for:			
	5 hours	1 day	3 days	1 week
Undiluted broth ¹⁾	4.4×10^6	4.5×10^6	4.6×10^6	4.3×10^6
Broth diluted 10-fold	1.9×10^6	2.5×10^6	1.3×10^6	1.0×10^6
Broth diluted 100-fold	7.1×10^6	3.8×10^6	2.7×10^6	1.8×10^6
Distilled water	1.8×10^7	8.3×10^6	8.8×10^6	8.6×10^6

¹⁾ Yeast extract (Balt. Biol. Lab., dehydrated), 1.0%; K_2HPO_4 , 0.2%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01%; Tween 80 (Atlas Powder Co.), 0.1%.

formation *per se* but to the injurious effect of concentrated solute, one should be able to duplicate this behavior in the absence of ice by subjecting the bacteria directly to concentrated solute. The use of broth of sufficient concentration to withstand solidification at -22°C ., therefore, should cause the cells to react at this temperature just as they would in a more dilute broth which had solidified. However, it was not considered profitable to proceed directly in this manner for several reasons. In the first place, it was anticipated that the preparation of a highly concentrated broth solution would not prove practical; secondly, one would still be dealing with an undefined system which could not be subjected, with advantage, to future critical study. Therefore, the use of a single, simple, pure compound was favored. Sodium chloride was selected because of its great solubility. Moreover, it might be expected that sodium chloride and other electrolytes would be present in broth and, after concentration concomitant with solidification, might exert a considerable lethal effect upon the cells. A 4.6 M (5.3 molal) solution

of sodium chloride was employed as the suspending medium in many of the experiments now to be summarized. Saline of this concentration will not freeze at $-22 \pm 2^\circ\text{C}$.

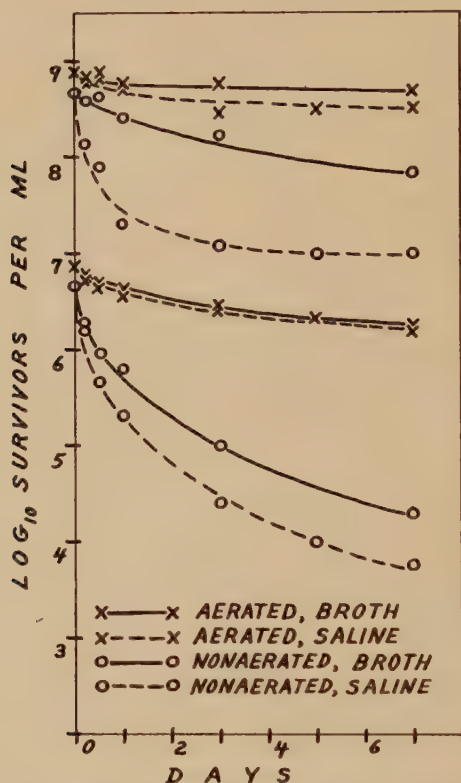


Fig. 1. Survival at -22°C . of aerated and nonaerated cells of *Escherichia coli* in frozen broth suspensions and in unsolidified 4.6 M NaCl suspensions.

Comparison of survival of bacteria in frozen broth suspensions and in unsolidified 4.6 M NaCl suspensions. Three features characteristic of the survival of *E. coli* in frozen broth are as follows. First, the survival curves have a continuously decreasing negative slope; second, percentage survival varies in proportion to the initial cell concentration; third, initial cultivation under forced aeration improves subsequent survival greatly (MAJOR *et al.*, 1955). These 3 features may be noted in figure 1 (solid lines). Aliquots also were suspended in 4.6 M NaCl and stored in an identical fashion. The survival curves from these unsolidified suspensions are likewise plotted in

figure 1 (broken lines). It will be noted that in all 3 respects survival in unfrozen saline is similar to that in frozen broth.

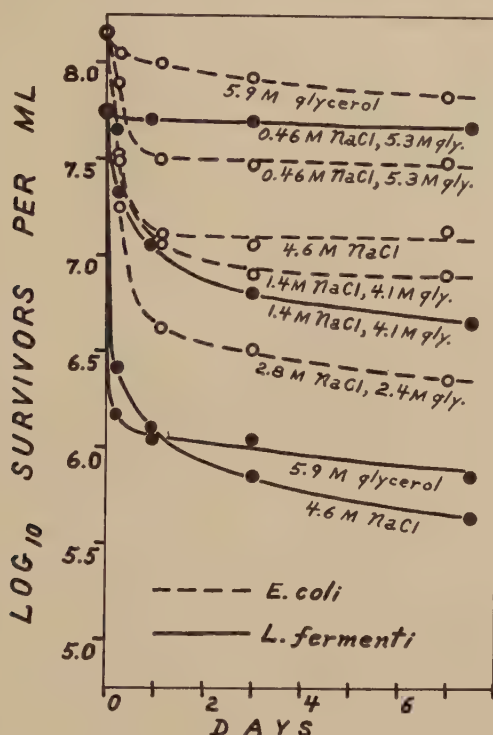


Fig. 2. The effect of glycerol upon the survival of bacteria in unsolidified saline at -22°C .

Effect of glycerol upon survival of bacteria in unsolidified NaCl suspensions. It has been shown that survival of bacteria (KEITH, 1913; HOLLANDER and NELL, 1954; SQUIRES and HARTSELL, 1955) and other microorganisms (MCENTEGART, 1954; SHERMAN, 1954) in frozen suspensions can be improved considerably by incorporating glycerol in the suspending medium. If death in frozen suspension is due to the lethality of certain concentrated solutes, then the protection afforded bacteria in frozen suspensions by glycerol must be due to the ability of this compound to counteract in some way the lethal effect of these solutes. If it could be demonstrated that glycerol counteracts the lethal effect of NaCl, this would provide further evidence that the saline test system used herein approximates the lethal conditions in frozen suspensions and, therefore, would provide additional indirect evidence that concentrated solute, not

ice *per se*, is the important lethal factor accompanying freezing.

A 5.9 M (13 molal) solution of glycerol will not freeze at $-22 \pm 2^\circ\text{C}$. Solutions of various glycerol:NaCl ratios were prepared by mixing together in different proportions 5.9 M glycerol and 4.6 M NaCl. The resulting glycerol-NaCl solutions will not solidify at -22°C . Cells were suspended in these glycerol-NaCl mixtures and survival at -22°C . was determined. The results from 2 experiments are presented in figure 2.

First, observe the survival curves obtained with *E. coli*. In those suspensions containing both glycerol and NaCl, the greater the ratio of the former to the latter the better the survival, and the suspension containing glycerol alone manifests the best survival. This experiment suggests that glycerol protects but does not prove this to be the case, since it could be claimed that the glycerol was inert and that improvement in survival was due to the decrease in concentration of NaCl. Therefore, short-term experiments were undertaken in which cells were suspended in one of the glycerol-saline mixtures and, also, in the respective concentration of each compound alone. Table 2 (*E. coli*) illustrates that the suspension containing glycerol with NaCl manifests greater survival than the suspension containing NaCl alone. The glycerol, therefore, would appear to be counteracting some of the lethality of the NaCl. This effect will undoubtedly be more pronounced after a longer storage period; however, it was not possible to hold the 1.4 M NaCl suspension in the supercooled state for longer than 1 1/2 hours.

With *L. fermenti* survival is not maximal in glycerol alone. In fact, death in 5.9 M glycerol is of the same magnitude as in 4.6 M NaCl (figure 2). Survival in glycerol is poor unless a trace of electrolyte is present (0.05 M NaCl is sufficient for maximum survival in 5.9 M glycerol). Except for this, *L. fermenti* reacts in the manner already described for *E. coli* (figure 2). Likewise, short-term experiments show that the improvement in survival with increasing glycerol-NaCl ratios is due to the protective action of the former rather than the mere decrease in concentration of the latter. In table 2 it will be noted that survival in 1.4 M NaCl has been improved 3-fold when glycerol in a concentration of 4.1 M is present.

Survival of *L. fermenti* upon successive exposures to 4.6 M NaCl. When bacterial suspensions in broth are repeatedly frozen and thawed, the survival curves resulting therefrom form a characteristic pattern (HARRISON, 1955). In order to see if cycling cells between

TABLE 2.

Survival of bacteria at -22° C. in unsolidified suspensions containing a mixture of glycerol and NaCl, glycerol alone, and NaCl alone.

Escherichia coli

Initial viable cell count per ml: 9.3×10^7

Menstruum	Viable cell count per ml after $1\frac{1}{2}$ hours
4.1 M Glycerol, 1.4 M NaCl	6.9×10^7
4.1 M Glycerol	9.2×10^7
1.4 M NaCl	3.7×10^7

Lactobacillus fermenti

Initial viable cell count per ml: 3.9×10^7

Menstruum	Viable cell count per ml after 1 hour
4.1 M Glycerol, 1.4 M NaCl	3.1×10^7
4.1 M Glycerol	8.0×10^6
1.4 M NaCl	1.0×10^7
4.6 M NaCl	1.2×10^7

concentrated solute and diluent would approximate the observed effect of repeated freezing and thawing, the technique summarized below was employed.

A suspension of *L. fermenti* in concentrated solute was prepared in the usual manner and dispensed in 7 ml aliquots into each of 4 centrifuge tubes. These tubes were designated no. 1, 2, 3, and 4. Plate counts were made from tube no. 1 after various storage intervals at -22° C.; from these counts a curve representing survival upon a single exposure to concentrated solute was prepared. After storage for an allotted period, tubes 2, 3, and 4 were removed from the cold chest and the unsolidified contents centrifuged at $10,000 \times G$ for 15 minutes. The supernatant from each of the 3 tubes was transferred to an empty tube and placed back in the cold chest. The cells in each of the 3 centrifuge tubes were suspended in diluent (0.5 g trypticase¹) and 0.6 ml of phosphate buffer solution²)

¹) Pancreatic digest of casein (Balt. Biol. Lab., dehydrated).

²) 14.8 g KH_2PO_4 and 68.5 g K_2HPO_4 per liter.

per liter), were again centrifuged for 15 minutes, the supernatant pipetted off, then the cells were resuspended in the original concentrated solute (from the cold chest), and the tubes were placed in the cold chest at -22°C . In this manner, the contents of these 3 tubes were subjected to a second exposure to concentrated solute. Plate counts were made from tube no. 2 after various storage intervals; from these counts a curve representing survival upon a second exposure to concentrated solute was prepared. After the allotted interval, tubes 3 and 4 were again removed from the cold chest and the unsolidified contents were manipulated as before. Plate counts were then made from tube no. 3 in order to prepare a curve representing survival after a third exposure to concentrated solute. Finally, tube no. 4 was manipulated again as outlined, and from this tube a curve representing survival after a fourth exposure was prepared (In order to monitor these experiments, a plate count was made from each tube immediately before and after each centrifugation, and also from each supernatant).

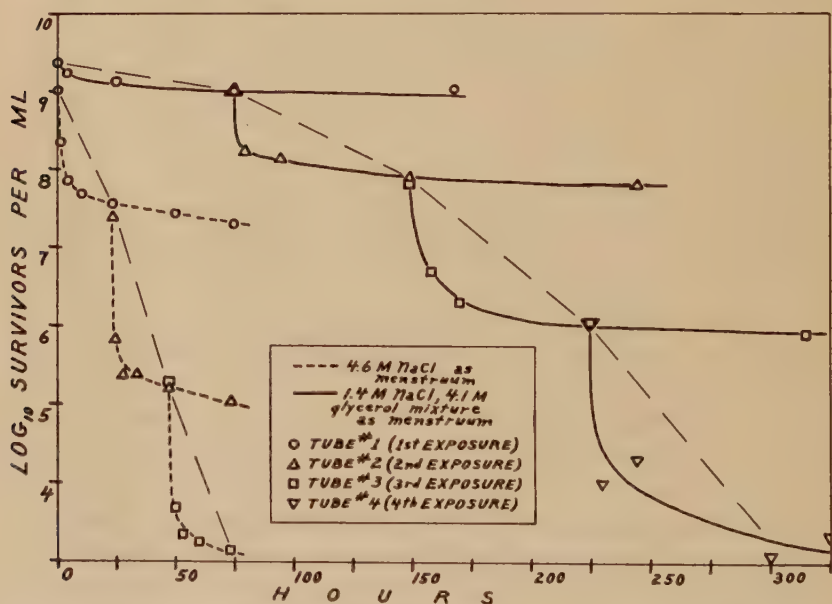


Fig. 3. Survival of *Lactobacillus fermenti* at -22°C . upon successive exposures to concentrated solute.

The results from 2 experiments are presented in figure 3 (The counts obtained immediately prior to each successive exposure to

concentrated solute have been connected with a broken line as an aid in showing their relationship to one another). These curves should be compared with those in figures 2A and 3 in a previous paper (HARRISON, 1955). It will be noted that in both cases a series of pendent curves is obtained. Each succeeding time the cells are exposed to concentrated solute they produce the same sort of survival curve as they do to each succeeding freezing.

Effect of volume of the suspension upon survival. In test tubes of a given diameter the survival of *E. coli* in frozen and unfrozen suspensions (table 3) is proportional to the volume of the suspension. This is probably a function of the surface area to volume ratio of the suspension rather than volume *per se* because, if equal volumes of suspension are dispensed into test tubes of different diameters, survival is inversely proportional to the tube diameter. It appears that some component of the atmosphere affects survival. This may account for the death which occurs in frozen distilled water suspensions.

TABLE 3.

Survival of *Escherichia coli* at -22°C . in suspensions of different volumes.

Menstruum	Volume of suspension	Initial viable cell count per ml	Viable cell count per ml after	
			1 week	4 weeks
Distilled water	5.0 ml	1.6×10^9	—	3.1×10^8
	0.2 ml		—	4.4×10^7
4.6 M NaCl	5.5 ml	1.5×10^9	3.6×10^8	—
	0.5 ml		2.1×10^8	—

Intracellular ice is not a lethal factor at -22°C . Experiments to demonstrate this are to be summarized elsewhere.

WEISER and OSTERUD (1945) attempted to determine the death rate during the actual process of solidification. They concluded that death was not due to indirect effects of extracellular ice formation such as concentration of solutes, "since in the latter case the maximum death rate reached at the completion of ice formation should continue at a constant rate. Contrary to this, the death rate was observed to diminish upon completion of ice formation." Their supposition with regard to the behavior of bacteria in concentrated solute is not borne out by the present research, since, as

has been demonstrated herein, cells exposed directly to concentrated solute do not die at a constant rate but die at a continuously decreasing rate — just as WEISER and OSTERUD observed. Later in their paper they admit that after ice formation is complete concentrated solute may be a lethal factor. They referred to this as “storage” death. Death by crushing, which they believe occurs during ice formation, they refer to as “immediate” death. The possibility of delineating accurately between “immediate” death during the “final stages of ice formation” and “storage” death following immediately thereafter is doubtful, since concentration of solute and formation of ice occur simultaneously. Had WEISER and OSTERUD plotted survival curves, it is probable that they would have obtained smooth curves of a continuously decreasing negative slope, the same sort of curves as seen in the figures herein. Any delineation of 2 distinct phases in the lethal process on such curves would be strictly arbitrary.

It is beyond the scope of the present paper to discuss the possible mechanisms whereby concentrated solute injures bacteria. Plasmolysis and/or protein denaturation are obvious possibilities. However, a number of interesting parallels between the effect of freezing-thawing on bacteria and on some inanimate systems might be mentioned. For example, just as repeated freezing-thawing kills bacteria, repeated freezing-thawing has been observed to denature bacterial protein (HAINES, 1938) and also to inactivate enzymes (KIERMEIER, 1947). Furthermore, the protective action of glycerol against freezing-thawing and concentrated solute is paralleled by its protective action on viruses (where it has been used as a preservative even at temperatures above freezing; Amer. Public Health Assoc., 1948) and on muscle contractile proteins (SZENT-GYÖRGYI, 1949). Glycerol also affords some protection against thermal denaturation of the proteins of rabbit serum and egg albumin (BEILINSON, 1929). Another property of glycerol worthy of note here is its ability to penetrate readily into cells as compared with other compounds, such as electrolytes, which penetrate relatively poorly (references given by HÖBER, 1945).

Conclusions and summary.

The lack of good evidence in support of a mechanical cause of death, together with the demonstrated similarities between the

behavior of bacterial cells in frozen broth and in unfrozen solute, has led to the consideration that death in frozen suspensions has to do with unsolidified solute. Concomitant with freezing, water is withdrawn from the suspending medium as ice, and the cells become subjected to concentrated solute. The degree of lethality of this unsolidified solute is, of course, dependent upon the compounds of which it is composed. At -22°C . sodium chloride is strongly lethal. Glycerol, on the other hand, may cause little if any death. In fact, glycerol is even able to counteract to some extent the lethality of sodium chloride. Another factor is involved in death at low temperatures; this is some component of the atmosphere. However, the primary cause of death in frozen suspensions appears to be solute which is concentrated concomitantly with the solidification of the suspension. These conclusions pertain only to bacteria of the type studied. In the case of cells of higher organisms, although the conclusions just presented may hold to a degree, death due to disruption of the cells by ice may be a factor.

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ISOLATION OF FORMALDEHYDE AND GLYOXYLIC ACID DURING THE OXIDATION OF ACETATE AND GLYCOLATE BY YEAST CELLS IN PRESENCE OF PHENYLHYDRAZINE

by

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(Received June 6, 1956).

The isolation of formyl, in the form of the carbonyl carbon of benzaldehyde, during the oxidation of acetate and glycolate by the respiration of living yeast cells in presence of phenylhydrazine has been reported in a preceding paper (BOLCATO, 1956). This result has led us to suppose, that an intermediate of the oxidation process may be formaldehyde, as was first observed for *E.coli* (BOLCATO, BOSCHETTI and MONTROYA, 1956). The present report deals with the isolation of this aldehyde in experiments with yeast cells similar to those referred previously (BOLCATO, 1956), and data are presented which indicate that formaldehyde is the precursor of the formyl group. Along with formaldehyde, also glyoxylic acid has been isolated as intermediate in the oxidation of acetate and glycolate.

METHODS.

Full details of the procedures employed in the experiments reported in this note have been described in a previous paper (BOLCATO, 1956). It is to be noted that the highest amounts of formaldehyde and glyoxylic acid were obtained when the respiration occurred at pH about 4 and 4—6 hours after the addition of phenylhydrazine.

Estimation of formaldehyde and glyoxylic acid.

The increasing formation of these intermediates during the process

could be observed by means of the following method: 50—100 ml of the filtrated medium, acidified with H_2SO_4 up to a final concentration of 1%, was steam distilled until a sample of the distillate no longer gave the specific colour with the chromotropic acid reagent. In the distillate formaldehyde was determined according to the method used by EDWARDS and KELLIE (1954). The determined formaldehyde was derived from the phenylhydrazones of the aldehyde and glyoxylic acid, as the latter is decarboxylated under these conditions.

When 50—100 ml of the same medium, made alkaline to pH 8.5, was steam distilled only the formaldehyde phenylhydrazone was decomposed, so the formaldehyde could be determined in the distillate independently of the glyoxylic acid present in the medium. The amount of glyoxylic acid was calculated by multiplying with 2.5 the difference between the amount of formaldehyde which was obtained from the acidic distillation and that which was obtained from the alkaline distillation. Control tests have demonstrated that this method gave a yield of about 50% of formaldehyde and glyoxylic acid present in the medium.

Isolation of formaldehyde.

At the end of the experiment the cell suspension was filtrated, the filtrate made alkaline to pH 8 and then continuously extracted with ether for 12 hrs (1st extract). The ether was evaporated, the residue dissolved in 3—4 ml of ethyl alcohol, poured into 100 ml of a 1% aqueous H_2SO_4 solution and steam distilled until a sample of the distillate gave no turbidity with some drops of a sulphuric solution of 2,4-dinitrophenylhydrazine (2,4-DNP). Formaldehyde was identified in the distillate according to the procedure used in similar experiments with *E.coli* (BOLCATO, BOSCHETTI and MONTÓYA, 1956).

Isolation of glyoxylic acid.

The medium, after the preceding alkaline extraction, was acidified with H_2SO_4 to congo red and submitted to an additional extraction with ether for 12 hrs (2nd extract). The ethereal extract was shaken with 20 ml of water, to remove the residual acetic or glycolic acid, and then again shaken with two small portions of 1 N Na_2CO_3 . By acidification of the combined Na_2CO_3 layers a precipitate was formed of impure glyoxylic acid phenylhydrazone.

By recrystallization from 50% ethanol yellow green crystals were obtained melting at 142°C. No depression was observed on admixture with synthetic glyoxylic acid phenylhydrazone.

Since the glyoxylic acid phenylhydrazone was frequently contaminated by appreciable amounts of pyruvic acid phenylhydrazone a procedure has been worked out, to separate these products, based on an observed reaction between synthetic glyoxylic acid phenylhydrazone and 2,4-DNP. Adding an alkaline or alcoholic solution of the synthetic phenylhydrazone to an hot sulphuric solution of 2,4-DNP a precipitate of glyoxal 2,4-DNP osazone was formed. It seems very probable, that in the hot sulphuric solution glyoxylic acid is decarboxylated to formaldehyde which, according to von PECHMANN (1897), undergoes aldolcondensation yielding glycolaldehyde. In presence of excess of 2,4-DNP, the aldehyde is converted in the glyoxal osazone. A similar reaction was described by von PECHMANN (1897) for the synthesis of the glyoxal osazone from a mixture of phenylhydrazine and formaldehyde dissolved in acetic acid. Pyruvic acid phenylhydrazone treated under the same experimental conditions was transformed in the 2,4-DNP derivative easily separable from glyoxal 2,4-DNP osazone.

The procedure used was the following: the combined Na_2CO_3 extracts (see above) were poured with stirring into 30 ml of 1% 2,4-DNP in 5N H_2SO_4 previously heated at 90°C. The precipitate, which rapidly formed, was filtered after standing at 30°C. for a few hrs. It was then washed with water and repeatedly with 1 N Na_2CO_3 to dissolve the pyruvic acid 2,4-DNP. The precipitate was again washed with water and then with boiling 95% ethanol. The residue was dried and purified by dissolving twice in nitrobenzene and precipitated with ethanol. The product melted at 322°C. and no depression was observed on admixture with synthetic glyoxal 2,4-DNP osazone.

Direct formation of the glyoxal in our experiments has to be excluded for the following considerations: a) the osazone would be present in the first ethereal extract at pH 8; b) it cannot be extracted by Na_2CO_3 being insoluble in alkaline solution; c) the synthetic osazone treated with hot sulphuric solution of 2,4-DNP gives a product melting at 240°C. not yet clearly identified.

RESULTS.

Quantitative data.

The results of seven typical experiments are assembled in Table 1.

TABLE 1.

Formation of formaldehyde, glyoxylic acid and benzaldehyde during the oxidation of acetate and glycolate by yeast cells in presence of phenylhydrazine (Ph).

Analytical determinations	Control with 1 g/l of Ph acetate and without substrates		Acetate with 1 g/l of Ph acetate			Glycolate with 1 g/l of Ph oxalate	
pH initial	5.1	5.2	5.1	5.2	5.2	5.2	5.3
pH final	5.3	5.4	5.9	5.7	5.6	5.7	5.6
Benzaldehyde as 2,4-DNP mg/l	0.0	0.0	30	35	25	65	90
Formaldehyde as 2,4-DNP mg/l	0.0	0.0	3	3	4	4	5
Glyoxylic acid as 2,4-DNP mg/l	0.0	0.0	6	0	4	27	17

Formaldehyde from the oxidation of methyl alcohol by yeast cells.

In the preceding paper (BOLCATO, 1956) it was reported that the oxidation of methyl alcohol by yeast cells, in presence of phenylhydrazine oxalate, gave good yields of benzaldehyde. Assuming that formaldehyde is intermediate in the microbial oxidation of methyl alcohol (MÜLLER, 1932), we supposed that it would be possible to trap appreciable amounts of formaldehyde, together with benzaldehyde, in experiments with methyl alcohol as sole substrate. On the contrary, from these experiments, and also in those with acetate, only small amounts of formaldehyde were trapped and identified with the chromotropic acid reagent. The failure to trap larger quantities of formaldehyde is certainly due to the fact that the formaldehyde phenylhydrazone is attacked by the yeast cells, while in presence of an excess of phenylhydrazine it is partially transformed into benzaldehyde (Table 2). The same results have been obtained in experiments with *E. coli* (BOLCATO, BOSCHETTI and MONTOYA, 1956).

It seems very probable that the excess of phenylhydrazine is the source of the active phenyl group, which is immediately formylated by the formyl formed by the formaldehyde dehydrogenase system (STRITTMATTER and BALL, 1955), according to the mechanism previously reported (BOLCATO, 1956).

TABLE 2.

Transformation of formaldehyde phenylhydrazone by yeast cells.

75 g w.w. of baker's yeast were suspended in 3000 ml distilled sterile water and aerated for 15 hrs. The suspension was then divided in three equal portions:

- A) with 0.2 g freshly prepared formaldehyde phenylhydrazone dissolved in a small quantity of acetic acid, stirred with 100 ml warm water and the mixture added to the cell suspension. pH adjusted to 5.5 with NaOH.
- B) with 0.1 g formaldehyde and 0.35 g phenylhydrazine previously dissolved in 100 ml water, acidified with acetic acid, and immediately added to the cell suspension. pH 5.5.
- C) like B) and 1.5 g phenylhydrazine oxalate dissolved in 150 ml hot distilled water. pH 5.5.

Time of incubation with aeration, 12 hrs. Temperature 30° C.

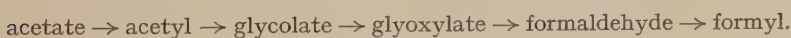
Analytical determinations		A	B	C
Formaldehyde as 2,4-DNP	mg/l	traces	traces	5
Benzaldehyde as 2,4-DNP	mg/l	3	5	70

Since formaldehyde itself directly yields the formyl group (in the form of the carbonyl carbon of benzaldehyde) this fact demonstrates that formaldehyde is the immediate precursor of the formyl in the process of acetate and glycolate oxidation by yeast cells.

As phenylhydrazine is unable to trap the formaldehyde firmly, we will study this problem with other trapping agents.

CONCLUSION.

The isolation of glyoxylic acid, formaldehyde and formyl as common intermediate products of the oxidation of acetate and glycolate, permits us to suggest the following metabolic pathway:



From the data now available one cannot establish whether these intermediates are concerned with synthetic or respiratory processes. As they are formed by a suspension of resting yeast cells one cannot exclude the latter possibility.

In support of our results it is to be remembered that CHALLENGER *et al.* (1927) and BERNHAUER and SCHEUER (1932) have observed the formation of glycolate from acetate in experiments with *A. niger*.

WEINHOUSE (1951), with the same mold, allowed to metabolize labeled acetate in presence of un-labeled glycolate found that appreciable radioactivities were incorporated in the isolated glycolic acid. Recently the glycolic acid oxidase (TOLBERT *et al.*, 1949; ZELITCH and OCHOA, 1953; KUN *et al.*, 1954) and the glyoxylic acid reductase (ZELITCH, 1955) have been isolated in plants and in mammalian tissues.

Summary.

During the oxidation of acetate and glycolate by the respiration of living yeast cells, in presence of phenylhydrazine, it has been possible to isolate glyoxylic acid, formaldehyde and formyl-compounds as intermediate products.

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ON THE DISINFECTION OF CATHETERS, CYSTOSCOPES, BRONCHOSCOPES AND SIMILAR INSTRUMENTS BY MEANS OF A MIXTURE OF ETHYLENE OXIDE AND CARBON DIOXIDE

by

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(Received April 5, 1956).

Generally speaking, disinfection by means of steam, whether under pressure or not, is to be preferred to other methods. In certain cases, however, the use of steam is impracticable, e.g. when one wishes to disinfect rubber and plastic catheters and instruments equipped with an optical device (cystoscopes, gastroscopes, bronchoscopes, etc.), as these would be damaged by steam. In these circumstances one must consider the use of a chemical disinfectant. However, many of these disinfectants are toxic and have to be thoroughly removed after their application, before the instrument can be used for man; others, again, act on the instruments one wishes to disinfect. As frequent handling increases the risk of the disinfected instruments being contaminated, it is necessary to use a method that makes it possible to disinfect the instruments, packed in a suitable bag without damage; after which it can be stored and, if necessary, transported in a sterile condition. Such a method we have found in the application of gaseous ethylene oxide (EO).

The possibility of disinfection by means of EO has been known since SCHRADER and BOSSERT (1936) and GROSS and DIXON (1937) applied for patents.

Much has already been published on the application of this chemical, a disinfectant action having been established with respect to many kinds of bacteria, fungi and yeasts and also some viruses (VERHOEVEN, 1947; PHILLIPS and KAYE, 1949; PHILLIPS, 1949; KAYE, 1949; KAYE and PHILLIPS, 1949; GINSBERG and WILSON,

1950; WILSON and BRUNO, 1950; KLARENBECK and VAN TONGEREN, 1954).

ETHYLENE OXIDE.

Ethylene oxide is a fluid which boils at 10.8°C . and freezes at -111.3°C . and can, if cool, be kept in the liquid state in cylinders, from which it can be liberated as a gas. Gaseous EO is highly inflammable and forms explosive mixtures with air in any proportion from 3 to 80%. Therefore EO is unsuitable for general use as it is. The risk of an explosion or fire can be obviated by mixing EO with at least 7.15 times its volume of carbon dioxide (JONES and KENNEDY, 1930).

EO is but slightly toxic and agrees in this respect with ammonia. In view of the results of animal tests ZERNIK (1933) assumes that a concentration of 1 mg/l could only cause abnormalities and eventually death, when inhaled for hours at a stretch.

The EO content of gas mixtures can be quantitatively determined by means of the method described by PHILLIPS and KAYE (1949), a certain quantity of the gas mixture being absorbed in a saturated solution of magnesium bromide in 0.1 n of sulphuric acid, the non-converted sulphuric acid then being titrated with 0.1 n of sodium hydroxide, with methyl orange as an indicator (1 ml of sulphuric acid used corresponds to 4.4 mg of EO).

APPARATUS.

The apparatus we used consisted of a metal cylinder fitted with a delivery device and filled with a mixture of 10% EO and 90% CO_2 ; a disinfection vessel in which the instruments were put; a manometer; a strike-back flask and a water-pump (see Fig. 1). The object of the delivery device is to obtain a gas mixture of a constant composition. If the gas mixture is allowed to flow straight from the cylinder, the accompanying cooling retards the evaporation of EO, so that before long almost pure CO_2 escapes. The insertion of the delivery device enables one to transfer a small amount of the liquid EO- CO_2 mixture present in the cylinder to the delivery vessel, after which the contents of the latter have to be used up completely. Therefore the capacity of the delivery vessel has to be adapted to the capacity of the vessel that is intended for use in the disinfection. In our experiments the delivery vessel contained about 10 l of gas mixture, the capacity of the disinfection vessel being about 3 to 5 l.

The disinfection vessel was a cylindrical glass jar, commensurate to the instruments we wished to disinfect, fitted with a rubber stopper carrying a glass tube provided with a stop cock. The introduction of a strikeback flask between apparatus and pump serves to prevent the flowing back of water into the disinfection vessel as a result of sudden changes in the pressure of the tap-water.

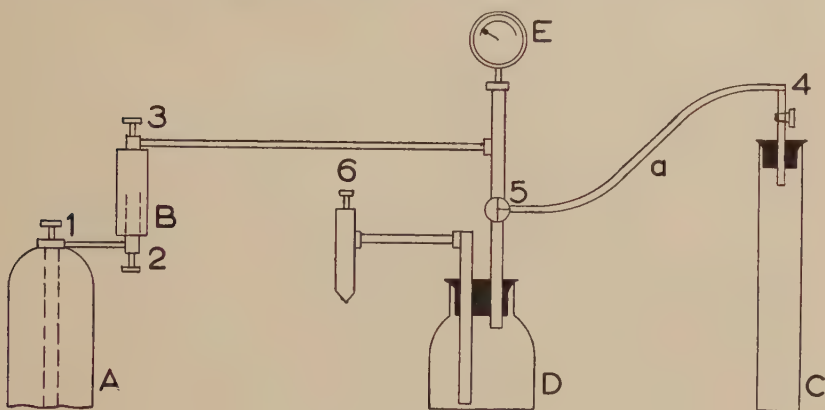


Fig. 1.

METHOD.

The basis of our disinfection method with the EO—CO₂ mixture is, that the air is evacuated as completely as possible from the vessel containing the instruments, by means of the water pump, after which the vessel is filled with the EO—CO₂ mixture. Methods have been described in which the EO is applied under pressure, but we have found the penetrative power of EO to be greater in a vacuum.

For this purpose stop cock 1 of the cylinder (A) and stop cock 2 of the delivery device (B) are turned open; both stop cocks being turned off again after 5 minutes. In the mean time the liquid EO—CO₂ mixture has filled the delivery device. The water-tap 6 is opened, three way cock 5 is placed in the vertical position and stop cock 4 of the disinfection vessel (C) is opened. When the manometer (E) indicates a vacuum of about 15 mm, the three way cock 5 is placed in the horizontal position and stop cock 3 of the delivery device is turned open until the vacuum has been cancelled.

This process of creating a vacuum and then neutralizing this with EO—CO₂ mixture is repeated once. Stop cock 4 of the disinfection vessel is then turned off, the rubber tubing a is detached from this vessel and the vessel itself is placed for a certain period

at a certain temperature. After the removal of the disinfection vessel stop cock 3 of the delivery device is turned open and the remaining gas is allowed to escape.

The objects with which we experimented were catheters of varying thickness (ureter-catheters) and composition (rubber, poly-vinyl-chloride, polyethelene, nylon, silicone), a cystoscope and a bronchoscope. Wide catheters and the last-mentioned two instruments were infected by immersing them completely in a jar of broth, which was then inoculated with the test-bacteria. After 24 hours' incubation at 37° C. the instruments were taken out of the broth-cultures, which had grown satisfactorily. Catheters with a narrow lumen were filled with a 24 hours' broth-culture by means of an injection-needle. After the infection the excess liquid was drained off. So all the objects of our experiments were highly infected with bacteria both inside and outside. The instruments were then put in cellophane bags, open at one end, placed in the disinfection vessel and exposed to the action of the EO—CO₂ mixture.

After having been disinfected the catheters were cut to pieces and each piece was put in a tube containing broth; the separate parts of the cystoscope and the bronchoscope being put in flasks filled with broth. The media were then incubated at 37° C. for 5 days and the growth was checked. When the broths were found to remain sterile, they were inoculated with the same test-strains in order to ascertain whether they really formed a suitable medium. In every case, 24 hours' incubation at 37° C. produced a satisfactory growth.

In other experiments the cellophane bag containing the instruments was sealed after the disinfection, e.g. by searing the open end between two small glass plates, whereupon it was placed at room temperature for 2 or 3 weeks. The bag was then opened and the sterility of the instruments checked.

We considered that the disinfection with the EO—CO₂ mixture ought to come up to the same bacteriological requirements that disinfection plants in which the disinfection is carried out with steam and formaldehyde must fulfil (BEKKER and ONVLEE, 1955) *viz.*, the killing of the test bacteria *E. coli* (resistance < 1 minute at 100° C.), *Staphylococcus aureus* (resistance < 1 minute at 100° C.) and three non-pathogenic, aerobe, sporogenous bacteria with resistances of > 2 < 3 minutes at 100° C., > 3 < 4 minutes at 100° C. and > 4 < 5 minutes at 100° C., respectively.

RESULTS.

In our first experiments we tried out the action of the EO—CO₂ mixture at room-temperature. We found that no reproducible results could be obtained even after the mixture had been allowed to act for 24 hours, so that there was no certainty that all the test bacteria had been killed at the end of this space of time. Reproducible results were, indeed, obtainable with an action period of more than 24 hours, but this is too long a time for practical purposes. However, what these experiments did show was that the action rate of the EO—CO₂ mixture increased, when the room-temperature rose.

Subsequent experiments were therefore made in the incubator at a temperature of 37° C. A great number of experiments showed that at this temperature the EO—CO₂ mixture was always able to kill all the test bacteria, even the most resistant spores, provided the action-period was at least 8 hours. It should be borne in mind that the instruments we used had been very highly infected, far more so than will ever be the case in practice.

Besides those with the test bacteria already mentioned, experiments were also carried out with a B.C.G. culture. These acid-fast bacteria were also found to have been killed by the EO—CO₂ mixture after an 8 hours' action at 37° C.

As stated above, we also carried out experiments in which the instruments, after having been disinfected, were placed at room-temperature for 2 or 3 weeks. At the end of this period the instruments were removed from the cellophane bags and their sterility was checked in the manner outlined. All the instruments were found still to be sterile. In addition to our experiments with the instruments mentioned above, we also made some with artificially infected rubber gloves; in these, too, disinfection was effected by the action of the EO—CO₂ mixture after 8 hours at 37° C.

A slight amount of moisture was found to be necessary for a reliable disinfection. Lyophilized cultures of bacteria and colonies transferred to slides and dried, were not killed by the above method. In practice, the instrument applied to man must be provisionally cleaned and rinsed with water or saline, drained to remove the excess liquid, packed in cellophane bags and disinfected.

In our experiments, which extended over more than twelve months and in which we sometimes used higher action temperatures and undiluted EO, we never observed any damaging effect on rubber, metal, lenses and luting.

DISCUSSION.

In many hospitals and sanatoria there is urgent need of a simple and reliable disinfection that does not damage fragile and often expensive instruments. This problem can be solved by using ethylene oxide.

This method of disinfecting has the following advantages:

1. the apparatus and the method used are comparatively simple;
2. the disinfection is very reliable, for it fulfils high bacteriological requirements;
3. if packed in cellophane bags, the instruments can be stored in the bags for a considerable time and, if necessary, be transported;
4. the instruments are not damaged by the disinfection process;
5. the risk of fire or explosion is eliminated by the addition of CO_2 to the EO.

The acting principle of EO is not yet fully known. It is probably based on the fact that the free carboxyl-, amino-, sulfhydryl- and hydroxy-groups in the protein are alkylated, a hydrogen atom being replaced by the hydroxyethyl radical (FRAENKEL-CONRAT, 1944). In support of this view it may be observed that other substances with alkylating properties also have a bactericidal effect (e.g. methyl bromide), which in some cases, is even greater than that of EO (e.g. ethylene imine). As these substances often have the disadvantage of being toxic, inconstant or difficult to prepare, their practical application is, as yet, out of the question.

S u m m a r y.

An apparatus and a method are described for a simple and reliable disinfection of catheters, cystoscopes, bronchoscopes, etc. by means of a gas mixture consisting of 10% ethylene oxide and 90% carbon dioxide. The air is evacuated by suction from a disinfection vessel containing the instruments and replaced by the ethylene oxide-carbon dioxide mixture, which must be allowed to act for 8 hours at 37° C. After disinfection, the instruments which have previously been packed in cellophane bags can be stored and transported sterile for a considerable period.

Bacterial spores with a resistance of 5 minutes at 100° C. are killed by this method, which has no damaging effect on the instruments.

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